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Sampling of Nanoliter Volumes of Mammalian Subcutaneous Tissue Fluid and Ultra-micro Flame Photometric Analyses of the K and Na Concentrations

By

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Abstract

HALJAMÄE, H *Sampling of nanoliter volumes of mammalian subcutaneous tissue fluid and ultra micro flame photometric analyses of the K and Na concentrations* Acta physiol scand 1970 78 1—10

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fluid was 4 67 [illegible] was 153 4 meq/l, the corresponding plasma val[illegible]pectively The average individual potassium tissu[illegible] 1 0) and the sodium tissue fluid plasma ratio 1 [illegible] Possible sources of error at sampling and analyses have been evaluated The potassium and sodium content of the local tissue fluid was higher than expected of an ultrafiltrate of plasma according to the generally accepted Gibbs Donnan constant (0 96) for these ions The apparent deviation of the obtained values from the Gibbs-Donnan constant can be explained by assuming that charged anionic macromolecules in the ground substance of the tissues affect the cation distribution in the local tissue fluid

The electrolyte distribution between the different fluid compartments of the body is still not completely known The fluids of the intravascular compartment lymph transudates exudates and transcellular secretions are fairly easy to sample and have been subjected to analysis (Greene *et al* 1931 Perlmann Glenn and Kaufman 1943, Folk Zierler and Lilienthal Jr 1948 Manery 1954 Elkinton and Danowski 1955 Yoffey and Courtice 1956 Rusznyak Foldi and Szabo 1967) The composition of tissue fluid, however is usually derived by indirect methods In most cases it is calculated assuming a Gibbs Donnan equilibrium exists across the capillary wall On the other hand Thorn (1960) discussed the possibility of the existence of a more fixed' fluid phase in the tissues together with a more movable fluid phase The presence of macromolecules and structural components with charged groups in the tissue spaces may affect the inorganic composition of the local tissue fluid Catchpole *et al* (recently reviewed by Chvapil 1967) have been working on the electro chemical properties of the colloids and on the equilibrial constants of different ions

in the ground substance of tissues From determinations of dilution potentials information was obtained about the density of negatively charged immobile colloids of the ground substance and of the mobility of ions The authors found that tissues with a high content of aggregated colloids (*e g* cartilage) had a potassium concentration ten times higher than in the blood while loose connective tissue with a high water content and low content of colloids had a potassium content close to the level in blood They also found that the composition of the interstitial phase may be affected by such factors as the state of local cellular metabolism For instance the existence of products from cell metabolism which are exchanges through the local fluid phase of the connective tissue spaces to capillaries or lymphatics, may affect the distribution of ions (Joseph Engel and Catchpole 1961) Rusznyak, Földi and Szabó (1967) do not think that cellular metabolism affects the composition of the tissue fluid and they believe that the inorganic composition of tissue fluid instead reflects the composition of plasma

There is thus evidence suggesting the existence of a fluid phase in the tissues the ionic composition of which is affected by local colloidal substances and substances involved in cellular metabolism Therefore it would be of interest to obtain direct measurements of the ionic composition of this fluid phase and especially of the ions involved in the sodium pump *i e* sodium and potassium In the present report local subcutaneous tissue fluid has been sampled and analyses of the potassium and sodium concentrations of the nanoliter volumes obtainable have been performed and compared to the concentrations of these ions in blood plasma

Attempts to sample tissue fluid and to determine its composition have been performed previously In 1938 Maurer isolated extracellular muscle fluid from frogs by inserting a thin capillary tubing lengthwise into the muscle He found a chloride serum extracellular fluid ratio of 0 993 and a significantly lower protein content in the tissue fluid Similar experiments were performed by Creese D Silva and Shaw (1962) who sampled interfibre fluid from guinea pig muscle The composition of this fluid was also significantly different from blood serum with a ratio of the ^{24}NaCl distribution value of fluid serum of 1 02 and with a much lower protein content of the interfibre fluid The use of implanted reservoirs for continuous sampling of interstitial fluid seems useful for further analysis of tissue fluid and for getting further information of the relations of the electrolyte distribution between the different fluid compartments (*e g* Burke 1964)

Methods

Dogs of mixed breed and both sexes and albino rabbits weighing 1400—2000 g were used

Tissue fluid sampling

[illegible] 30 40 mg/kg [illegible] of the [illegible] with [illegible] cular [illegible] with forceps and an incision through the epidermal and dermal layers made with a pair of small stainless steel scissors The cut was rapidly extended 2 to 3 cm following one of the subcutaneous tissue fasciae and the edges of the incision were immediately widely separated If a

slight bleeding at the bottom of the cavity occurred, a new incision was made to avoid contamination of the local fluid with blood constituents If there was a small bleeding from the subepidermal capillary plexus of the widely separated edges of the incision the vessel was coagulated electrically if no contamination of the bottom of the cavity seemed possible In order to keep the edges of the incision wide apart to avoid contamination of the bottom of the cavity with damaged epidermal cells or exudation from the damaged subepidermal capillary plexus, sutures were attached from the edges to a steel ring if necessary Immediately before the sampling of fluid the connective tissue fascia constituting the bottom of the cavity during these manipulations was incised and the edges widely separated so that the clean underlying fascia or sometimes the muscle fascia was exposed During all these manipulations it was important to keep the incision covered with liquid paraffin to avoid evaporation with resulting changes of the initial electrolyte concentration of the fluid

Micropipettes, drawn from thin capillary glass tubing, with thin pointed tips (20—60 μ) were used at the sampling of the fluid The pipettes were cleaned by washing with concen-

place during the brief time periods needed for tissue fluid sampling, when the pipettes were carefully cleaned as above Therefore, as a large number of pipettes were used it was easier to make them from ordinary glass than from quartz Immediately before the tissue fluid sampling, the pipette was partially filled with some liquid paraffin to provide an airtight upper seal By slight subpressure applied to the pipette during small movements of the tip of the pipette over the tissue of the central parts of the bottom of the incision small drops of fluid mixed with droplets of liquid paraffin could be obtained Before the removal of the pipette from the incision cavity the lower end of the pipette was also sealed with liquid paraffin

The contents of the pipette were emptied onto a quartz glass covered with liquid paraffin in a Petri dish Ordinary glass wares could not be used as it was not possible to get them absolutely clean of potassium and sodium which was necessary as a fairly long period of wet-

the bubble and the few cells present sedimentated onto the surface of the quartz glass during the time period of 15 to 30 min allowed to pass before further procedure It was possible to obtain a 20—50 nl (1 nl$=10^{-9}$ l) sample of fluid within a few min Usually 2 or more samples from different sites were taken from each experimental animal and in a few experiments several samples were taken from the same cavity

Blood samples were taken from a peripheral vein or the carotid artery into siliconized and heparinitized tubes under liquid paraffin After centrifugation the plasma samples were applied onto the same quartz glass under liquid paraffin as the tissue fluid samples The same type of pipettes as used for the tissue fluid sampling were used at the transfer of the plasma samples from the tubes to the quartz glass The same analytic procedure for nl sample was performed

Equipment for analysis of potassium and sodium

The samples were analyzed by an ultra micro flame photometric method (Haljamae and Larsson 1968) where the potassium and sodium concentrations of nl samples of solutions could be determined The sample to be analyzed was placed on the tip of a thin Pt Ir-wire and allowed to dry The wire holder was placed in a slit of the flame photometer and was pushed into the flame by a solenoid which was actuated by the registrating equipment The potassium and sodium em[illegible]
the flame and equipped w[illegible]
photocells were connected [illegible]
of the two elements of th[illegible]
of the samples were made from thin quartz glass tubing The calibration of the pipettes was performed according to the fluoremetric method described by Prager Bowman and Vurek (1965), using quinine hydrobromide in 0 1 N sulfuric acid Only pipettes with a variation of less than 2 per cent at calibration were used for the sample handling During the pipetting procedures the pipettes were kept sealed with liquid paraffin in the upper part so that no air contact with the sample was possible before the final application of the sample onto the wire The flame photometer was calibrated with standard solutions prepared from KCl and NaCl (Spectrographically standardised substance Matthey and Co Ltd London, England)

TABLE I Average tissue fluid (TF) and plasma (P) potassium and sodium concentration, dogs (n = 16) Several tissue fluid samples from different sites from the subcutaneous tissue of the hind leg and two different plasma samples were taken from each dog and each sample was separately analyzed

Dogs	Potassium meEq/l				Sodium meq/l			
	TF	P	TF P	TF/P	TF	P	TF P	TF/P
n = 16	4 67[1]	3 78	0 89[2]	1 25[3]	153 4[4]	142 3	11 0[5]	1 08[6]
S E M	±0 14	±0 11	±0 13	±0 04	±3 3	±2 5	±2 7	±0 02

[1] P < 0 001 vs plasma — [2] P < 0 001 vs zero — [3] P < 0 001 vs 1 00 — [4] P < 0 02 vs plasma. — [5] *P < 0 001 vs zero* — [6] *P < 0 001 vs 1 00*

Electrolyte analysis of the tissue fluid and plasma samples

The electrolyte analysis of the primary interstitial fluid sample was performed according to the following procedure With a calibrated quartz glass pipette a known volume (21 8 nl) of tissue fluid was taken from the lateral part of the drop and removed under the surface of the liquid paraffin to a new area of the quartz glass By taking the sample from the lateral part of the drop it was possible to obtain a sample free of paraffin droplets and containing only a few cells A known volume (141 4 nl) of 2 N nitric acid pro analysi was added The tissue fluid—2N nitric acid sample was allowed to stand for 120 min at room temperature in order to coagulate and wet ash the protein content After this time the proteins were not completel ashed but no change of the electrolyte content of the clear fluid part of the nitric acid—tissue fluid sample was observed when longer periods of wet ashing were used before analysis After the wet ashing a known volume (21 8 nl) of the ashed sample was removed to a new area of the quartz glass and diluted with a known volume (70 8 nl) of glass redistilled water These later dilutions provided the samples for potassium analysis but before the sodium analysis was performed a further dilution was made 21.8 nl of the diluted sample + 282 8 nl of distilled water The same procedure was used for the electrolyte analyses of plasma samples

Results

Average potassium and sodium concentrations of tissue fluid and blood plasma from 16 dogs are given in Table I The average potassium value for tissue fluid was 4 67 meq l S E M ± 0 14 and that for plasma 3 78 meq/l S E M ± 0 11 The average difference between the potassium concentration of tissue fluid and that of plasma was *0 89* meq *l significantly different from zero (P<0 001) The average* ratio potassium tissue fluid plasma was 1 25 S E M + 0 04 and significantly higher (P<0 001 than 1 00 The sodium concentration of tissue fluid was on the average 11 0 meq l higher than that of plasma P<0 001) The average sodium tissue fluid plasma ratio 1 08 was also significantly (P<0 001) different from 1 00 The tissue fluid plasma ratios for both potassium and sodium were thus higher than expected according to a Gibbs-Donnan equilibrium constant (0 96)

The variation between the potassium and sodium values for multiple tissue fluid samples from different sites of the hind leg from the same animal is exemplified in Table II Duplicate analyses of known nanoliter volumes of each sample were performed The difference between the values for these duplicate analyses was in

TABLE II Potassium and sodium concentrations of tissue fluid sampled from multiple sites from subcutaneous tissue of the hind leg from the same dog Corresponding values for the two separately prepared and analyzed plasma samples

Dog	Sample		Potassium meq/l		Sodium meq/l	
13	Tissue fluid	n=5		4 61		143 9
	S D			±0 12		±3 1
	Plasma	n=2	3 99	3 90	136 2	138 1
14	Tissue fluid	n=6		4 24		139 1
	S D			±0 15		±1 7
	Plasma	n=2	3 42	3 46	137.2	137 2

significant The variation between the potassium values of the tissue fluid samples from different sites was small average variation dog 13 was ±2 6 % and for dog 14 ±3 5 % The same was true for the sodium values average ±2 2 % and ±1 2 % respectively These variations include errors introduced by sampling pipetting procedures and the variability of the ultramicro flame photometer Little variation was also found between the plasma values of the two blood samples taken, each being prepared and analyzed separately

The effect of multiple sampling of fluid from the same liquid paraffin cavity of dogs and rabbits on the potassium concentration is presented in Fig 1 With time (0 – time – anesthesia) always a slight decrease of the potassium concentration of the local tissue fluid was obtained, which might indicate contamination with exudate due to local tissue irritation

To evaluate errors due to contamination from the glass wares used the effects of the liquid paraffin the 2 N nitric acid the quartz redistilled water and the variations at the pipetting procedures and the stability of the equipment for analysis the same procedure as used for tissue fluid and plasma samples was applied to standard

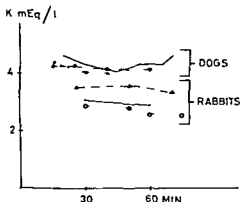


Fig 1 Potassium decrease of tissue fluid from dogs and rabbits when several samples were taken from the same liquid paraffin cavity at various times

solutions of known concentrations of potassium (100 ppm) and sodium (1000 ppm). The obtained average values from the analyses were for potassium 3.18 ppm S.D.± 0.11 (n=8) and for sodium 2.29 ppm S.D. ±0.22 and thus close to the theoretical values 3.14 and 2.25 respectively.

Discussion

Usually the electrolyte concentration of tissue fluid has been supposed to correspond to an ultrafiltrate of plasma (Gamble 1952). This assumption has been based on protein and electrolyte differences between lymph, transudates, oedema fluids etc. on one hand and plasma on the other hand (for literature survey see Yoffey and Courtice 1956, Rusznyák, Földi and Szabo 1967). When determining ultrafiltrability of serum electrolytes (*e.g.* Tarail, Hacker and Taymor 1952) or the electrolyte content of oedema fluids (Hastings *et al.* 1927, Folk, Zierler and Lilienthal Jr 1948) it must be remembered, however, that in the first case the effects of the cellular tissue and the intercellular substance is missing and that during oedema conditions the perfusion and hydration of the tissues and thus the ground substance is profoundly changed. Therefore such results may not be in accord with in situ values under normal physiological conditions.

The present results obtained for the potassium and sodium concentrations of local tissue fluid are not completely in accord with those expected according to a Gibbs-Donnan equilibrium with the plasma electrolyte content. The significantly higher potassium values of the local tissue fluid as compared to plasma can not only be explained by a profound cellular damage at the sampling of the fluid. The subcutaneous tissue fascia or the muscle fascia from which the fluid was sampled are relatively acellular and with the precaution taken a possibility for contamination of the fluid with cells from the epidermal layer seems small. If, however, such a damage were a dominant factor for the higher potassium content of tissue fluid a high variation in the potassium content would also be expected between tissue fluid samples from different sites of the hind leg of the same animal as the same degree of cellular injury could not be expected during the different samplings. The variation of 2.5–3.5 % of the potassium content during such multiple sampling from different sites (cf. Table II) is well within the variation of 3.5 % obtained when using the same sample preparation of standard solutions. The obtained variation for tissue fluid also includes possible errors due to capillary irritation with consequent exudation and contamination of the sample with a few cells. When several samples were taken with long time intervals from the same cavity a slight decrease of the initial potassium [illegible] concentration was obtained as expected due to the vascular irritation. As the sam[illegible] in the different sites normally were taken within a few minutes and as th[illegible] labelled albumin into the obtained fluid was slow (Haljamäe and Fre[illegible] published) there is no evidence for exudation being a significant source of [illegible] sample content. Some cells, a few leukocytes and a few red blood cells m[illegible] the potassium values. Even if a high theoretical contamination of the [illegible] leukocytes is supposed, e.g. 8 al. (×300 cu mm) with

an average diameter of 10 μ thus roughly corresponding to a volume of 500 μ^3 per cell and with an intracellular potassium concentration 150 meq/l then the total leukocyte volume would correspond to 1.5×10^{-3} of the total sample volume This would affect the tissue fluid potassium by an increase of approximately 0.2 meq/l As however, most of the cells in the primary sample were allowed to sedimentate before the sample to be ashed and analyzed was taken the expected effect even of such a heavy contamination must be considerably smaller A contamination of the sample with a few red cells will have an insignificant effect on the obtained potassium concentration as the potassium content of dog red cells is only a few meq/l higher than that of plasma A possible slight hemolysis of the blood or leakage from the red corpuscules during the preparation of the plasma sample will therefore not affect the obtained potassium ratio between fluid and plasma significantly

The sodium content of the tissue fluid was also higher than expected if contamination with intracellular fluid took place during the sampling Any contamination with red blood corpuscules or leukocytes both having a sodium concentration lower than that of plasma would also decrease the real sodium value A slight hemolysis will however, decrease the sodium content of plasma somewhat as in spite of the high sodium content of dog red blood corpuscles the sodium concentration of plasma is higher The obtained electrolyte values for dog plasma were in accordance with previous reports (*e g* Carr and Schloerb 1959) and no significant variation between the electrolyte content of the duplicate separately prepared plasma samples was obtained (*cf* Table II)

The protein content of a tissue fluid is considered to be significantly lower than that of plasma (Maurer 1938 Drinker 1946 Creese D Silva and Shaw 1962 Rusznyak Foldi and Szabo 1967) This has also been observed in the tissue fluid obtained with the presented liquid paraffin cavity method A low protein content and a slow exchange of labelled protein from the intravascular compartment to the obtained fluid was demonstrated (Haljamae and Freden to be published) The average plasma tissue fluid ratio was 3 1 and the labelled albumin content 2 hrs after injection was in the tissue fluid only 8 % of the simultaneous plasma content This suggests that the obtained fluid is not an active exudate due to capillary injury because in such a case a high protein content and a rapid exchange of labelled protein to the fluid could be expected If the potassium and sodium concentrations are corrected for the volume occupied by non aqueous proteinaceous material then the potassium tissue fluid plasma ratio of 1 25 would be a few per cent lower 1 18 (if plasma protein 6—7 % and thus tissue fluid protein about 2 %) and the sodium tissue fluid plasma ratio would be 1 03 after correction

Differences between the content of ions other than potassium and sodium may also exist between the obtained tissue fluid and plasma Therefore differences in the potassium and sodium concentrations between the two fluids could be due to difference in interference from such ions at analysis Such interference effects on the potassium and sodium readings of the used ultramicro flame photometer were evaluated by adding known concentrations of calcium magnesium phosphate bi-

carbonate and proteins to standard solutions. All the above substances with the exception of protein decreased the indicator readings for both potassium and sodium by 3—4 % (Keesey 1968, Haljamäe and Larsson 1968). Thus if there is a higher concentration of these ions in the local tissue fluid due to the cellular metabolic activities in the surroundings, the obtained values for tissue fluid potassium and sodium will be somewhat lower than the real and decrease the real tissue fluid plasma electrolyte ratios. The protein content of plasma and tissue fluid, as judged from the interference experiments, will give an increase of 2—4 % of the indicator readings (Keesey 1968). If in spite of the wet ashing and dilutions used, there is interference from proteins at the analyses of the samples, this would also lower the real tissue fluid plasma electrolyte ratios due to the higher protein content of plasma. It may be concluded that the effect of possible differences in the distribution of protein and ions other than potassium and sodium will probably result in a decrease of the real tissue fluid plasma electrolyte ratios. Therefore even slightly higher ratios of both potassium and sodium may exist between these fluids than those observed with the analytic procedures employed.

The obtained results thus indicate the existance of a local tissue fluid with a potassium and sodium concentration higher than that of plasma. If it is assumed that a Gibbs-Donnan equilibrium exists across the capillary wall it seems reasonable to believe that the anionic colloidal substances of the interstitial spaces may affect the movement and concentrations of cations. Preston, Davies and Ogston (1965) demonstrated that hyaluronic acid may markedly decrease the flow of water and thus immobilize interstitial fluid. Cotlove (1954) showed that the diffusion of inulin, sucrose and ³⁶Cl into rabbit tendon was slower than expected and hypothesized that the free diffusion was retarded due to the effect of the interstitial matrix. Similar diffusion delay of saline through connective tissue fascia was shown by Day (1952) who also demonstrated that treatment with hyaluronidase speeded up the diffusion considerably. Gated video micro-densitometer recordings of the diffusion of dyes through the interstitial space of frog mesentery by Widerhielm (1966) gave a diffusion coefficient of only 1/50 of that for diffusion of the same dye in a gel. As recently concluded by Maners (1966) it is thus clear that polysaccharide aggregates in the connective tissues can act as physical barriers to diffusion.

The interaction of the mucopolysaccharides of the connective tissue with other ions has also been studied. Catchpole *et al.* (see Chvapil 1967) have in numerous investigations demonstrated an ion binding capacity of the polyelectrolyte colloids of the interstitial tissues. They found a selectively higher binding of potassium by the ground substance than of sodium (Catchpole, Joseph and Engel 1966). The results in the present investigation show a higher potassium tissue fluid plasma ratio (1.25) than the *corresponding sodium ratio* (1.08) which could be explained on the basis of selective potassium binding or immobilization by the ground substance. When considering the difference in *meq/l* the results indicate a higher sodium content in the obtained tissue fluid than of *potassium*. The potassium content of tissue fluid water is on the average less than 1 *meq/l* higher than that of plasma water

while the corresponding sodium value will be about 5 meq/l higher and if a Gibbs Donnan distribution is assumed this difference will be even higher

A higher sodium content would agree with the results of Dunstone (1960) who demonstrated a higher affinity of cartilage for sodium than for potassium Under the influence of oestradiol interstitial connective tissue binding of sodium in excess of chloride was shown by Hvidberg Jensen Holm and Langgård (1963) This hormone increases the connective tissue ground substance hence the results suggest a sodium binding capacity of the ground substance By inducing acidosis it was also demonstrated that at changed tissue pH a greater part of the excess connective tissue sodium could be released (Langgård Jensen Holm and Hvidberg 1963) Creese D Silva and Shaw (1962) also found a slightly higher content of $^{24}NaCl$ in interfibre fluid from guinea pig muscle than in plasma although no significant difference was obtained

The electrolyte content of the locally sampled nanoliter volumes of subcutaneous tissue fluid seems to be in accord with that of an expected more 'fixed' type of fluid the composition of which is affected by the ground substance in the interstitial spaces As a decrease of the sodium and potassium activities are caused by the anionic mucopolysaccharide substances and as alkali metal ions do not readily form stable complexes with other ions or molecules it seems reasonable that the interstitial fluid phase obtained will differ from that of a plasma ultrafiltrate The findings of Headings Rondell and Bohr (1960) also indicate a relatively loose binding of excess sodium by the substances They could show that by increasing the hydrogen or potassium concentration in an incubation medium the excess sodium in the inulin space of dog carotid artery could be replaced within a 3 min incubation Their results are in accord with previous reports which have demonstrated that mucopolysaccharide substances readily can behave as ion exchangers (*e g* Farber 1960) It has also been possible to show with the presented method that when tissue perfusion and hydration as well as normal cellular metabolism are disturbed (*effect* of hemorrhagic shock) there is a significant difference between local tissue fluid electrolyte changes as compared to those of blood plasma (Hagberg Haljamäe and Rockert 1968)

Further characteristics considering the protein content and exchange of the local tissue fluid obtained with the liquid paraffin cavity method will be presented in a separate report (Haljamae and Freden to be published)

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Proteolytic Inhibitors in Plasma from Man Treated with a Protease from Aspergillus oryzae

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Abstract

JURGENS, J, S LINDVALL, O MAGNUSSON and K ORTH *Proteolytic inhibitors in plasma from man treated with a protease from Aspergillus oryzae* Acta physiol scand 1970 78 11—19

The level of total heat stable and heat labile inhibitors of a proteolytic enzyme from Aspergillus oryzae—protease I—have been studied in plasma from eight patients After treatment of these patients by infusions of 150 mg of the proteolytic enzyme in 5 % levulose solution the change in the level of the different inhibitors have been estimated It has been shown that there is a lowering of the total inhibitors content after each infusion This decrease is only partly compensated for by the day following the first infusion After the forthcoming infusions the decrease is completely compensated for resulting in a level before each subsequent infusion which is lower than that before commencement of treatment This remaning decrease can be attributed to the heat stable inhibitor A possible mechanism for the thrombolytic effect of the protease is mentioned

The fibrinolytic factor from Aspergillus oryzae which was first disclosed by Stefanini *et al* (1959) was purified by Bergkvist (1963 a b and c) He found the main active principle to be a proteolytic enzyme, protease I which bore a close similarity to plasmin in many of its properties Bergkvist also found that the inhibition of protease I by serum showed a striking resemblance to that of plasmin (1963 d) On administering the enzyme intravenously into cat Bergkvist and Svard (1964) could establish that the amount of protease inhibitors in serum diminished after each injection of the enzyme Similar results have also been reported by Roschlau (1964 1965) on administration of an equivalent enzyme preparation to dogs

O Brien *et al* (1968) studied the inhibition of antiplasmin by protease I as well as the fibrinolytic effect of the enzyme on injection to patients with cancer and found that the blood content of antiplasmin as well as inhibitors to protease I sank after infusion of the enzyme Roschlau (1968) also found a decrease in the content of inhibitors of blood in a patient treated with the enzyme

During the elaboration of a method for determination of the amount of inhibitors of protease I in serum, Lindvall *et al* (1969) established that the inhibition was exerted by two main components As well as protease I one of these inhibits trypsin and the other plasmin The inhibitors could be determined separately by heating serum to 60° C Shulman (1952) has shown that at this temperature the trypsin inhibitor is unstable while the plasmin inhibitor is stable

Using this technique the present investigation has been carried out with a view to study in more detail the level of inhibitors in the blood after administration of protease I to man Thus, the total amount of inhibitors to protease I on the one hand and the amount of the two main inhibitors on the other have now been determined in plasma from patients with a thrombotic condition before and after i v infusion of the enzyme

Material and methods

Patients

Altogether 8 patients have been treated with the proteolytic enzyme from Aspergillus oryzae and sex age, weight and diagnosis of these patients can be seen in Table I

Treatment and sampling

The patients were treated ambulatorally and 150 mg of the enzyme, dissolved in 500 ml 5 % levulose solution was infused into the vena brachialis during the course of 1 hr

Citrate blood (1 ml 3.8 % sodium citrate solution + 9 ml blood) was taken from the same vein immediately before commencement and 15—30 min after completion of infusion

Proteolytic enzyme

Two different enzyme preparations from Aspergillus oryzae were studied These have been developed as a result of co-operation between Astra Sweden (protease I), the mainly used preparation and Connaught Medical Research Laboratories Canada (CA 7)

Protease I — The enzyme was prepared and purified according to the methods described by Bergkvist (1963 a)

It was used as a freeze-dried sterile and pyrogen free preparation in 50 ml vials containing 50 mg (lot 25035)

The proteolytic activity determined by a caseinolytic method according to Bergkvist (1963 a) was 17.0 ± 1.5 CU per mg

CA 7 — Preparation and purification of the enzyme has taken place according to method described by Ives and Tosoni (1967)

The enzyme was used as a freeze-dried sterile and pyrogen free preparation in 50 ml vials containing 50 000 Ca units (lots 1009, 1011)

The proteolytic activity determined according to a method of Dyer and Kadar (1964) is 1000 Ca units per mg Determination according to the caseinolytic method gives an activity of 17.0 ± 1.5 CU per mg

Estimation of the inhibitors

The inhibitors were estimated using methods described by Lindvall *et al* (1969) except that plasma was used instead of serum According to these methods the total inhibiting effect of plasma was measured by incubating for 5 min at 37.5° C 0.1 ml citrated plasma with 0.9 ml TRIS buffer containing > 10 µg protease I more than the assumed amount of inhibitor in the sample The proteolytic activity was then determined using a viscosimetric method The inhibitor level in the sample was obtained by subtracting the remaining proteolytic activity from the amount added and is expressed as µg protease I per ml plasma Differentiation of the inhibitors as heat stable and heat labile has been performed by heating the samples at 60° C

TABLE I Number of infusions and levels of total, heat stable and heat labile inhibitor in plasma of 8 different cases before treatment

Case	Day of first injection	Sex	Age Years	Weight kg	Diagnosis	Number of infusions	Inhibitor level in µg PI/ml plasma		
							Total	Heat stable	Heat labile
No 1 So I 2646/66	4/3 —66	Female	62	103	Thrombosis of central retinal vein	6	124	72	52
No 2 Ma Au 2505/66	16/3 —66	Male	74	68	Thrombosis of central retinal vein	6	137	68	69
No 3 Ja Al 2746 66	3/3 —66	Male	70	64	Thrombosis of central retinal vein	4	161	100	61
No 4 Fr Ha 2702/66	21/3 —66	Female	29	52	Post thrombotic *syndrome* with recurrent thrombophlebitis	3	173	88	85
No 5 He Chr 2996/66	10/3 —66	Male	68	72	Occlusion of the central retinal artery	3	161	94	67
No 6 We El 7205/65	9/12—65	Female	59	62	Cronic recurrent thrombophlebitis with varicosis	3	160	90	70
No 7 La W 3621/67	9/12—65	Male	22	68	Varicosis with signs of thrombo-plebitis	2	20[illegible]	105	97
No 8 Br No 2566/68	10/12—65	Male	22	8[illegible]	Occlusion of right *popliteal artery*	1	119	73	46
No 8 Br No 2566/68	11/3 —66	Male	22	8[illegible]	Occlusion of right popliteal artery	1	142	68	74

for d ffe ent t mes nd or m [illegible]

ase I

Results

As seen in Table I, values for the total amount of inhibitors of protease I in plasma from patients before treatment varied *from 119—202 µg protease I per ml plasma* The amount of heat labile inhibitor varied from 46—97 and that of heat stable from 68—105 µg protease I per ml plasma

During the elaboration of a method for determination of the amount of inhib of protease I in serum, Lindvall *et al* (1969) established that the inhibition exerted by two main components As well as protease I one of these inhibits try and the other plasmin The inhibitors could be determined separately by hea serum to 60° C Shulman (1952) has shown that at this temperature the try inhibitor is unstable while the plasmin inhibitor is stable

Using this technique the present investigation has been carried out with a vie study in more detail the level of inhibitors in the blood after administration of tease I to man Thus, the total amount of inhibitors to protease I on the one l and the amount of the two main inhibitors on the other have now been determ in plasma from patients with a thrombotic condition before and after i v infusio *the enzyme*

Material and methods

Patients

Altogether 8 patients have been treated with the proteolytic enzyme from Aspergillus o and sex, age, weight and diagnosis of these patients can be seen in Table I

Treatment and sampling

The patients were treated ambulatorally and 150 mg of the enzyme dissolved in 500 ml levulose solution was infused into the vena brachialis during the course of 1 hr

Citrate blood (1 ml 3 8 % sodium citrate solution + 9 ml blood) was taken from the vein immediately before commencement and 15—30 min after completion of infusion

Proteolytic enzyme

Two different enzyme preparations from Aspergillus orzyae were studied These have developed as a result of co-operation between Astra Sweden (protease I), the mainly preparation and Connaught Medical Research Laboratories Canada (CA 7)

Protease I — The enzyme was prepared and purified according to the methods describe Bergkvist (1963 a

It was used as a freeze dried sterile and pyrogen free preparation in 50 ml containing 50 mg (lot 25055)

The proteolytic activity determined by a caseinolytic method according to I kvist 1963 a was 17 0 ± 1 5 CU per mg

CA 7 — Preparation and purification of the enzyme has taken place according to met described by Ives and Tosoni (1967)

The enzyme was used as a freeze dried sterile and pyrogen free preparatio 50 ml vials containing 50 000 C_2 units (lots 1009, 1011)

The proteolytic activity determined according to a method of Dyer and K 1963 is 1000 C_2-units per mg Determination according to the caseino method gives an activity of 17 0 ± 1 5 CU per mg

Estimation of the inhibitors

The inhibitors were estimated using methods described by Lindvall *et al* (1969), except plasma was used instead of serum According to these methods the total inhibiting effec plasma was measured by incubating for 5 min at 35 5 C 0 1 ml citrated plasma with 0 TRIS buffer containing 5 10 µg protease I more than the assumed amount of inhibito the sample The proteolytic activity was then determined using a viscosimetric method inhibitor level in the sample was obtained by subtracting the remaining proteolytic act from the amount added and is expressed as µg protease I per ml plasma Differentiation of inhibitors as heat stable and heat labile has been performed by heating the samples at 6(

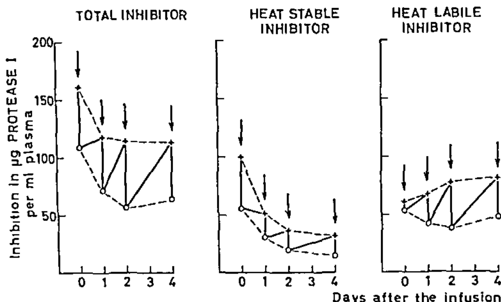


Fig 3 Inhibitor changes in case 3 after infusions of 150 mg protease I Inhibitor level before (+) and after treatment (○) The arrows indicate day of treatment

On dividing the total amount of inhibitors in plasma into heat labile and heat stable inhibitors the observed decrease in the total amount of inhibitor after the first infusion can essentially be ascribed to a decrease in the level of heat stable inhibitor, as is apparent from the figures Consequently the level of this inhibitor remained low after the initial decrease Only after a pause in treatment did the value for this inhibitor reach approximately the same level as that before treatment

The heat labile inhibitor was affected to only a slight extent at the first infusion On the other hand, an obvious decrease in this inhibitor took place at the subsequent treatments the decrease being totally compensated for by the time of the following treatment During the course of the series of treatment this compensation even brought about a slight increase in the level of heat labile inhibitor but after a pause of 13 days the value for the heat labile inhibitor had returned to that shown before the commencement of treatment

The second patient who was treated on 6 different occasions (case 2) received first 4 infusions of solutions containing CA 7 and subsequently two treatments with protease I It can be seen from Fig 2 that a decrease in the total inhibitor amount per ml plasma also occurred with this patient as a result of the treatments The resulting decreases have not wholly compensated for between the treatments but a gradual decrease in the total amount of inhibitor in the patient s plasma could be demonstrated during the course of the series of treatment The recovery after the first infusion was nevertheless less than after the others As is apparent from the figure the heat stable inhibitor was most affected also in this case, but when a

TABLE II Changes in inhibitor level of plasma from 5 cases treated with aspergillus protease

Case	Day of infusion	Treatment	Inhibitor in µg per ml plasma					
			Total	Change	Heat stable	Change	Heat labile	Change
		Before	173		88		85	
	21/3 —66			52		37		15
		After 150 mg CA-7	121		51		70	
No 4		Before	145		75		70	
Fr Ha	22/7 —66			41		29		12
2702/66		After 150 mg CA-7	104		46		58	
		Before	131		55		76	
	23/3 —66			30		13		17
		After 150 mg CA-7	101		42		59	
		Before	161		94		67	
	10/3 —66			63		44		19
		After 150 mg Protease I	98		50		48	
No 5		Before	118		50		68	
He Chr	11/3 —66			49		25		24
2996 66		After 150 mg Protease I	69		25		44	
		Before	118		41		77	
	12 3 —66			26		18		8
		After 100 mg Protease I	92		23		69	
		Before	160		90		70	
	9 12—65			31		47		+16
		After 150 mg Protease I	129		43		86	
No 6		Before	117		68		49	
We El	10 12—65			55		44		11
7205 65		After 150 mg Protease I	62		24		38	
		Before	104		35		69	
	14 12—65			58		20		38
		After 150 mg Protease I	46		15		31	
		Before	202		105		97	
	9 12—65			58		51		7
No 7		After 150 mg Protease						
La W		I	144		54		90	
3621 67		Before	152		67		85	
	13/12—65			50		26		24
		After 150 mg Protease I	102		41		61	

Case	Day of infusion	Treatment	Inhibitor in µg per ml plasma					
			Total	Change	Heat stable	Change	Heat labile	Change
		Before	119		73		46	
	10/12—65			20		34		+14
No 8		After 150 mg Protease						
Br No		I	99		39		60	
2566/68		Before	142		68		74	
	11/3 —66			51		33		18
		After 150 gm Protease						
		I	91		35		56	

had sunk to values under 50 µg per ml plasma the heat labile inhibitor changed to a greater degree at the subsequent infusions

Case 3 received 4 infusions of protease I As is seen in Fig 3, a decrease in the total amount of inhibitor per ml plasma occurred after each occasion of treatment also in this case The resulting recoveries, as in the previously described cases were such that the value after the first treatment and subsequently was at a level lower than that before treatment The initial decrease in the total amount of inhibitor can also be ascribed to a decrease in the heat stable inhibitor When this decrease had taken place the heat labile inhibitor was affected to a greater extent in the infusions which followed

In Table II, the results from patients who had received 2—3 infusions are accounted for In all cases a decrease in the total amount of inhibitor per ml plasma occurred in connection with the first infusion the decrease being essentially ascribed to a change in the heat stable inhibitor Otherwise the pattern is in agreement with the results obtained in the cases accounted for previously In one of the cases (case 5), the dose has been lowered to 100 mg at the final treatment As seen the resulting decrease of the total amount of inhibitor was lower On differentiating between the inhibitors it could be established that the heat stable inhibitor was affected most while the heat labile inhibitor was affected to only a slight extent

Discussion

During studies on inhibitors to protease I in serum of healthy subjects Lindvall *et al* (1969) established that the total amount of inhibitor corresponds on average to 146 µg (range 106—202 µg) protease I per ml serum Of this amount 60 µg (range 38—80 µg) was comprised of a heat labile and 86 µg (range 53—133 µg) of a heat stable inhibitor In the present investigation the values given for these inhibitors in patients with thrombotic conditions before treatment do not deviate appreciably from these values Assuming that the blood volume is ca 70 ml/kg b w , a dose of 150 mg of the protease should not exceed any of the inhibitors at the first infusion and the results show also that no such exceeding of an inhibitor is evident

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As is apparent from the results, an immediate decrease in the total amount of inhibitor takes place at the first infusion This change can be ascribed to a decrease in the heat stable inhibitor When this has sunk to a low value, the subsequent infusions affect the heat labile inhibitor to a greater extent and the latter is restored between each of the treatments as a rule to the same value as that before the infusion According to Lindvall *et al* (1969), the last mentioned inhibitor is common to protease I and trypsin and the results indicate that this inhibitor has a rapid resynthesis The heat stable inhibitor, which has an inhibiting effect against plasmin as well as protease I according to the same authors, is on the other hand, not regenerated so rapidly In fact results from case 1 indicate that a long time is required in order to restore the level of antiplasmin to the same value as that before treatment During investigations on a_{-} macroglobulin the fraction of which in serum has antiplasmin activity Nihlen and Ganrot (1967) also came to the result that this fraction dit not quite recover its original concentration during the observation period (up to 16 days) after an intravenous infusion of streptokinase to man

The obvious decrease observed at the first infusion as well as the results from the case which received a lower dose indicate that the protease is first bound in vivo to the heat stable inhibitor which the protease has in common with plasmin Only subsequently is the activity of the heat labile inhibitor made use of Therefore, in vivo there appears to exist a preference for formation of an enzyme inhibitor complex between the heat stable antiplasmin inhibitor and the protease as opposed to the formation of a complex between antitrypsin and the protease

On discussing the results of studies on the inhibitors in vitro Lindvall *et al* (1969) advanced a hypothesis for the thrombolytic activity of protease I in vivo which has been demonstrated by Bergkvist and Svard (1964) Roschlau and Tosoni (1965) and Jurgens (1966) It was considered that the mechanism for the effect of protease could probably be that the enzyme deprives the inhibitor of plasmin from inhibiting physiologically activated plasmin by means of complex formation with it The results obtained in this investigation show that the strong affinity of protease I for antiplasmin causes a decrease in the latter and thus can be considered to support the hypothesis

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Relations of Polyuria and Polydipsia in Experimental Diabetes Insipidus

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Abstract

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Hypothalamic radio-frequency lesions were produced in unanesthetized goats with the use of thermo-couple electrodes permanently implanted into the median eminence region. Lesions restricted to the median eminence induced the triphasic response typical of experimental diabetes insipidus (DI). The polyuria of the temporary phase of DI developed before the polydipsia commenced. The increased water intake was apparently secondary to the excessive renal water loss. When the median eminence lesions were extended into the midhypothalamic region no clear distinction could be made between the onset of polyuria and polydipsia. Similar lesions encroaching upon the anterior hypothalamus and affecting the paraventricular nuclei caused temporary hyperdipsia but no transient polyuria, or a much delayed temporary increase in urine flow. All three types of lesions induced the permanent phase of DI after a latent period of 6 to 11 days. The polydipsia of the permanent phase occurred after the commencement of the polyuria (observations in 2 animals) and was evidently due to a physiological stimulation of the thirst mechanism.

Median eminence lesions which abolish the neural control of the release of antidiuretic hormone (ADH) from the neurohypophysis result in a typical triphasic response (Fisher, Ingram and Ranson 1938). Within few hrs after the injury polyuria and polydipsia commence. This temporary phase of experimental diabetes insipidus (DI) *lasts for a few days and is followed by an interphase of about a week's dura*tion. During the interphase the fluid exchange remains at a subnormal level and the urine becomes highly concentrated due to an excessive and uncontrolled release of ADH from the degenerating neurohypophysis (O'Connor 1952, de Wied 1966). When the neurohypophyseal storage of ADH is emptied, polyuria and polydipsia reappear (the permanent phase of DI). The intensity and the duration of the permanent phase depend on the extent of the median eminence lesion. Rather soon after the lesioning a release of ADH may start from neurosecretory fibre endings in temporarily damaged parts of the median eminence. The amounts of ADH released in this manner may be sufficient to prevent the manifestation of overt DI. Therefore only the temporary phase of DI may be seen after a complete transection of the

supraoptico-hypophyseal tract below the median eminence (*cf* Coggins and Leaf 1967)

It is well established that median eminence lesions performed under anesthesia may induce polyuria before the animals have drunk any water (Richter 1935 and others) Polyuria was also found to precede polydipsia after radio-frequency lesioning of the median eminence in two unanesthetized goats (Gale 1964) Other studies, however, have indicated that the polydipsia may not always be rigidly secondary to polyuria in experimental DI (*cf* Wolf 1958, Smith and McCann 1964) The present study of experimental DI in the goat indicates that the time relation between the onset of polydipsia and polyuria may depend upon the extent and the localization of the hypothalamic injury

Methods

Six adult female goats (b w 30—40 kg) were used In the following the individual animals are indicated PI PII MI, MII and AI AII with regard to the extent of their hypothalamic lesions (see results) The goats were confined in metabolism cages from the day after the brain implantation and until they were sacrificed They had free access to water and chaffed hay and received about 400 g of commercial grain mix (with 6 g of NaCl added) each afternoon However in goat AI a temporary post lesion restriction of the food and water supply had to be made to prevent dangerous overhydration and overeating Unless a more frequent recording of the fluid exchange was made, water intake and urine output were routinely measured at 8 a.m and 5 p m every day

Brain implantations In all goats a pair of thermocouple electrodes were implanted bilaterally into the median eminence region under general anesthesia as described by Gale (1963) With the 3 or 4 mm uninsulated distal ends of the electrodes penetrating the median eminence the electrode tips were placed 4 mm apart close to the sphenoid bone on both sides of the infundibular stem The direction of the electrodes through the brain varied between the animals The most posterior approach to the median eminence was made in goat PI (through the habenular region and via the anterior border of the corpus mammillare) The most anterior approach was made in goat AI In this animal the electrodes passed through the anterior commissure and close to the posterior border of the optic chiasma

Induction of diabetes insipidus One to 8 weeks after the implantation the thermocouple electrodes were used to produce lesions in the median eminence region of the goats By passing radio-frequency energy between the uninsulated distal ends of the electrodes the temperature of the electrode tips was raised to 70° C for 5 min The lesioning was made without anesthesia The animals had their urinary bladder catheterized and were standing in the metabolism ... ition ... the ... thus

Samples Blood samples were obtained in heparinized syringes from the jugular vein During the first 24 to 48 post lesioning hrs urine samples were collected at intervals via a retention catheter inserted into the urinary bladder Urine was sampled in this manner also during the initial part of the permanent phase of DI At other times the urine was collected separated from feces) in a bucket placed ...

Analyses Plasma and urine ... an EEL flame photometer and the Cl ... method of Brun (1949) For determination ...

Histology The animals were killed by decapitation under Nembutal anesthesia Perfusion through the carotids was rapidly performed with physiological saline followed by 5 % formol saline Due to the lesioning of the infundibular stem the pituitaries were only loosely attached to the brains and had to be embedded and sectioned separately After formol fixation a block of the brain including the diencephalon was embedded in celloidin and cut in serial transverse sections at 30 μ The brain sections were stained with tuloidine blue The pituitary was embedded in paraffin and cut in serial sagittal sections at 5 μ Some of the pituitary sections from goats AI and PI were stained by the method of Gomori (1941) The remaining sections were azan stained according to Mallory Heidenhain *cf* Koneff 1938)

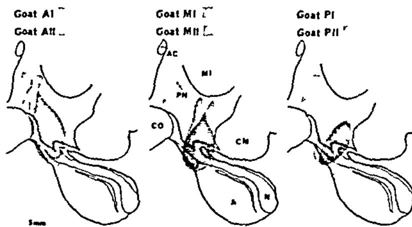


Fig 1 Sagittal projections of the hypothalamic lesions

A = Adenohypophysis
AC = Anterior commissure
CM = Corpus mammillare
CO = Chiasma opticum
MI = Massa intermedia
N = Neurohypophysis
PN = Paraventricular nucleus

Results

Brain and pituitary injuries

In all goats the lesions were found to involve the ventral portion of the median eminence and to penetrate the infundibular stem A complete interruption of the neural connection between the hypothalamus and the neurohypophysis had occurred causing widespread degenerative changes in the neural lobe tissue No Gomori positive material could be detected in the neural lobes of the two Gomori stained pituitaries goats AI and PI As earlier observed in the goat and other species (Adams Daniel and Prichard 1963) the damage to the infundibular stem had also resulted in an infarction involving most of the anterior lobe tissue No alterations were observed in the intermediate lobes

The main part of the brain lesion involved the tissue between and 1 5—2 mm in front and behind the uninsulated distal ends of the electrodes Apparently due to insufficient insulation a narrow strip of tissue between the nearest insulated parts of the electrodes had also become involved in the lesion in all animals except in goats PI and PII giving the lesions a pear shaped appearance in sagittal projection Thus the localization and the extent of the lesions was to a high degree determined by the direction of the electrodes and by the effectiveness of their insulation (Fig. 1) Examination of the brains indicated that the character of the temporary phase of DI in the individual animal was correlated to the localization of the hypothalamic injury

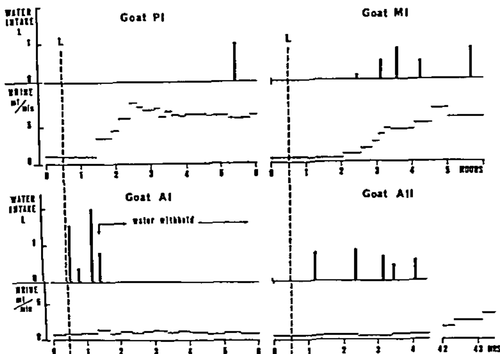


Fig 2. *Above, left* Commencement of the temporary phase of diabetes insipidus in a goat with a lesion restricted to the median eminence Polyuria precedes polydipsia

Above, right Simultaneous onset of polyuria and polydipsia in a goat with a median [illegible]

[illegible]), or with much delayed

[illegible] ching upon the anterior

[illegible]

L = Production of the lesion

The temporary phase of diabetes insipidus

Since the lesioning of the brain was performed in the unanesthetized animal having free access to water and having the urinary bladder catheterized it was possible to study the immediate effect on the water exchange of the goats Three different patterns of the temporary phase of DI could be distinguished

1) *Polyuria preceding the polydipsia*

The polyuria preceded the polydipsia in goats PI and PII with lesions involving the ventral and posterior parts of the median eminence and the anterior part of the corpus mammillare (Fig 1 right) In goat PI the urine flow gradually increased from 1 ml/min to a level between 6 and 8 ml/min during the 2nd post-lesioning hr During this period urine osmolarity dropped from 600 mosm/l to about 90 mosm/l The animal did not drink, however, until 4 1/2 hr after that the lesion was made (Fig 2 above, left) The plasma osmolarity was 303 mosm/l half an hour before

drinking commenced as compared to a pre lesion level of about 295 mosm/l. In goat PII a similar rise in urine flow (0.8 to 5.5 ml/min) and a marked fall in urinary electrolyte concentration occurred between 1 1/2 and 2 1/2 hr after the median eminence had been injured. Significant drinking did not take place until 30 min later. No determinations of plasma osmolarity were made in this animal. The temporary phase of DI lasted for about 2 days in goat PI and for 4 days in goat PII.

2) *Simultaneous onset of polyuria and polydipsia*

In the two goats (MI and MII) with lesions extending into the mid hypothalamic region (Fig. 1, middle), no clear distinction could be made between the commencement of polyuria and polydipsia. In goat MI the rise in urine flow occurred during the 2nd and 3rd post lesioning hr. During this period the animal drank 1.5 l of water. The polyuria had a tendency, however, to start before the drinking in this goat (Fig. 2 above right). In goat MII the tendency was the reverse. This animal drank 1.1 l of water in two sequences just before the period of urine collection during which the first obvious rise in urine flow was observed (2 hrs after the hypothalamus had been injured). The duration of the temporary phase of DI was about 2 days in these goats.

3) *Polydipsia without or with much delayed polyuria*

The two goats (AI and AII) with lesions extending into the anterior hypothalamus and involving the paraventricular nuclei (Fig. 1 left) did not exhibit any typical temporary phase of DI. Five min after the radio frequency heating of the hypothalamus was completed goat AI (having the most anterior extent of the lesion) started to drink. Within the first post lesioning hr 4.5 l of water were consumed. Marked haemolysis developed and the water was withheld in order to prevent dangerous overhydration (Fig. 2 below left). The abnormal thirst persisted for the two following days during which the water intake was restricted to 7 l/day (about 3 times the normal intake of this goat). On the 3rd post lesioning day plasma osmolarity had dropped to 265 mosm/l and plasma Na to 125 meq/l indicating marked overhydration. From then on water was no longer restricted since the water intake dropped below pre lesioning control level and remained low until the onset of the permanent phase of DI. The abnormal thirst occurred together with conspicuously increased appetite. To prevent overeating the food intake was restricted to 1 1/2 times the normal for this goat during the first 4 days after the hypothalamus had been injured. In spite of the initial overhydration and the complete interruption of the infundibular stem no transient polyuria developed (Fig. 2 below left). Before the onset of the permanent phase of DI the maximum urine flow recorded was 2.4 ml/min. This level of urine flow was reached on the 4th post lesioning day. Even then the urine osmolarity did not fall below 450 mosm/l.

The response of goat AII to the hypothalamic damage was similar to but less dramatic than of goat AI. During the first 3 hrs after the lesioning this goat drank 2.5 l of water but urine flow remained at <0.5 ml/min. No hyperphagia appeared

in conjunction with the polydipsia A moderate transient polyuria did not develop until 40 hrs after the neural connection between the hypothalamus and the pituitary had been interrupted (Fig 2 below, right) The polyuria lasted for about 24 hrs

The permanent phase of diabetes insipidus

The time interval between the radio-frequency heating of the median eminence and the appearance of the permanent phase of DI was rather constant in the 6 goats (6 to 11 days) In two of the animals (MI and AI) the permanent phase commenced during normal working hours making it possible to study the time relation between the onset of polyuria and polydipsia In both goats the polyuria developed before water intake increased over the interphase levels During the night before the beginning of the permanent phase, goat MI drank 400 ml of water and had a urine flow of 0 7 ml/min The urine osmolarity was 1100 mosm/l Between 8 and 9 a m urine flow rose to 5 ml/min while urine osmolarity dropped to 300 mosm/l No drinking occurred until 9 30 a m The plasma osmolarity and Na^+ concentration were not increased significantly over normal levels during the commencement of the permanent phase The same was true at later stages during the DI of this goat

In goat AI (having shown temporary post lesioning hyperdipsia but no transient polyuria) the permanent phase of DI appeared in the morning of the 7th post lesioning day Between 9 a m and 1 p m urine flow increased from 1 6 to 5 7 ml/min and urine osmolarity dropped to 170 mosm/l The goat which had not drunk any water over night, did not start drinking until 3 p m that day During the permanent phase of DI in this animal plasma Na remained above 150 meq/l and plasma osmolarity above 310 mosm/l which indicated chronic dehydration

All goats except MI were sacrificed within 3 weeks after the commencement of the permanent phase No significant decline of the polyuria was observed in these animals Goat MI was studied for 2 months after the beginning of the permanent phase During the 2nd month urine flow gradually declined below 2 ml/min concomitant with a rise in urine osmolarity to about 400 mosm/l

Discussion

The post lesioning course of the water turnover in goats PI and PII confirms the earlier observation (Gale 1964) that a typical triphasic experimental DI may be induced also in this species by lesions restricted to the median eminence In these two animals the initial transient polydipsia did not begin until polyuria had developed and the polydipsia did not cause any sign of hemodilution Therefore the increased water intake observed during the temporary phase of DI by all probability was secondary to an excessive renal loss of water in goats PI and PII

The relations of water intake and output changed when the median eminence injury was extended to other parts of the hypothalamus In goats MI and MII with lesions affecting the midhypothalamic region no clear distinction could be made between the commencement of polyuria and polydipsia Polydipsia and a lack

of transient polyuria or polydipsia in combination with a much delayed increase in urine flow, characterized the acute response to the hypothalamic injury in goats AI and AII. In these animals a narrowing protrusion of the lesion encroached upon the anterior hypothalamus and affected the paraventricular nuclei. Post-lesioning hyperphagia also occurred in goat AI, and postprandial thirst may have contributed to the excessive drinking seen in this animal. However, the polydipsia developed before significant amounts of hay had been consumed and water was drunk in amounts causing hemolysis, marked plasma hypoosmolarity, and hyponatremia. This indicates that the injury in fact had induced primary hyperdipsia.

Lesions designed to induce DI were found to cause a water intake in nephrectomized rats which significantly exceeded that of nephrectomized controls (Smith and McCann 1964). It was suggested that the effect might be due to a destruction of an area in the medial hypothalamus which normally inhibits drinking. An alternative explanation was that the lesions induced polydipsia by some irritative effect on the hypothalamic drinking center. The latter appears to be the most likely explanation of the hyperdipsia observed in goats AI and AII. Their lesions encroached upon a region of the hypothalamus where electrical stimulation elicits drinking in the goat (Andersson and McCann 1955). Further, the hyperdipsia was transient and hypernatremia and plasma hyperosmolarity developed later during the permanent phase of DI in goat AI. This indicates that the final effect of the lesion was a decreased sensitivity of the hypothalamic thirst mechanism to its normal stimulus.

In spite of the marked polydipsia and a complete interruption of the neural connection between the hypothalamus and the neurohypophysis, transient polyuria did not develop in goat AI and was much delayed in goat AII. It suggests that a temporary unphysiological release of antidiuretic material occurred from the hypothalamus proper. The paraventricular nuclei were affected in both animals. A damage to the neurosecretory cells of these nuclei or to other parts of the hypothalamic neurosecretory system may have liberated ADH (or its precursor) in amounts sufficient to suppress or delay the transient phase of polyuria.

The relations of polyuria and polydipsia in the beginning of the permanent phase of DI were studied in two of the animals only (goats AI and MI). In both goats the polyuria was fully developed before the polydipsia started. Chronic dehydration was present during the permanent phase of DI in goat AI and plasma osmolarity values below normal level were never observed during the permanent phase in any of the six goats. This supports the general view that drinking during the permanent phase of DI is the result of a physiological stimulation of the thirst mechanism (*cf* Goggins and Leaf 1967).

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The Disappearance of Xe^{133} and I^{125} from Skeletal Muscle of the Anesthetized Dog during Sympathetic Cholinergic Vasodilatation

By

PER BOLME and LENNART EDWALL

Received 6 March 1969

Abstract

BOLME, P. and L. EDWALL. *The disappearance of Xe^{133} and I^{125} from skeletal muscle of the anesthetized dog during sympathetic cholinergic vasodilatation.* Acta physiol. scand. 1970. *78.* 28—38.

The disappearance rate of I^{125} and Xe^{133} from a depot in the gastrocnemius muscle of the dog was determined during vasodilatation caused by activation of the sympathetic cholinergic vasodilator nerves. During vasodilatation the disappearance rate of I^{125} and Xe^{133} increased. No systematic difference in the disappearance rate was observed between the water soluble (I^{125}) and the fat-soluble (Xe^{133}) tracer. The vasodilatation did not usually last far more than 30 sec and during this period the increase in disappearance rate often exceeded the increase in blood flow. The disappearance rate of Xe^{133} was studied from different muscles during stimulation of hypothalamic vasodilator pathways and was found to be enhanced from the splenius, latissimus dorsi and gastrocnemius muscles but not from the masseter muscle.

Vasodilator nerve stimulation and acetylcholine infusion caused comparable increases in disappearance rate during the blood flow increase. When the blood flow was back to resting levels, a decrease in disappearance rate below control values was seen. The increase in disappearance rate during sympathetic nervous vasodilatation was usually less than during metabolic vasodilatation and after metabolic vasodilatation no decrease was observed in the disappearance rate. The results support the hypothesis that during metabolic vasodilatation precapillary sphincters are opened up leading to an increased capillary surface area while the vasodilator nerves do not directly influence the exchange surface area.

Several investigations indicate that the sympathetic cholinergic vasodilator nerves innervate precapillary resistance vessels, i.e. small arteries and arterioles in skeletal muscle of cat and dog (Folkow, Mellander and Öberg 1961, Renkin and Rosell 1962, Bolme and Fuxe 1967). However, different effects are reported on the tissue blood exchange of solutes following vasodilator nerve activation. Hyman *et al.* (1959) observed in cats no change in the clearance of I^{131} from an intramuscular depot following hypothalamic vasodilator outflow stimulation. It was suggested that the sympathetic vasodilator fibres influence vessels not involved in nutritional exchange. On the other hand Barlow and Walder (1965) reported an enhanced disappearance

rate of Na^{24} from the gastrocnemius and soleus muscles of the cat upon activation of the vasodilator nerves, indicating that an increased tissue blood exchange was elicited

In view of these conflicting results the present study was performed to further investigate the tissue blood exchange following sympathetic vasodilatation The vasodilator nerves to the skeletal muscles of the dog were activated by hypothalamic or sympathetic lumbar chain stimulation and the disappearance rate of i m injected radioisotopes was measured Furthermore, the effect of sympathetic nervous vasodilatation was compared with the effects following vasodilatation due to acetyl choline infusion or motor nerve stimulation

Methods

Experiments were performed in 30 mongrel dogs weighing 7—24 kg When hypothalamic stimulation was to be performed the dogs were premedicated with morphine (0 5 mg/kg i.m) and anesthetized with α-chloralose (100 mg/kg i v) In other types of experiments the anesthetic was pentobarbital (30 mg/kg i v)

In most experiments *M gastrocnemius* was studied The preparation of the muscle varied. In some cases and always when the hypothalamus was stimulated no preparation of the muscle was performed and the i m isotope injections were made through a cutaneous incision In these experiments the blood flow to the entire hind limb was measured with an electromagnetic flowmeter (AB Eledia Stockholm Sweden) by a probe fitted on the femoral artery In other dogs the gastrocnemius muscle (alone or in some experiments together with the posterior tibialis muscle) was isolated from surrounding tissues and the blood flow to the muscle was measured by the electromagnetic flowmeter or by a drop recorder Lindgren 1958) In the latter case heparin (1000 IU/kg i v) was given to the dog and the vein from or the artery to the muscle was cannulated The arterial pressure was measured in all experiments by a Statham pressure transducer (P 23 AC) Heart rate was recorded in some experiments by an ordinate writer fed with the pressure signal (Goldschmidt and Lindgren 1962 The recordings were made on a Grass model 5 polygraph A branch of *A poplitea* was cannulated and used for i a infusions In order to avoid movements the prepared limb was immobilized by screws in the tibia and femur In some experiments the neuromuscular blocking agent gallamine iodide (Flaxedil®) was given (1 5—2 mg/kg i v) to block the muscular movements In those experiments the animals were given artificial respiration

In a few experiments disappearance measurement was made from muscles other than gastrocnemius Those were *M masseter*, *M latissimus dors* and *M splenius* and none of the muscles were isolated

Vasodilatation was sometimes elicited by stimulating the hypothalamic vasodilator nervous outflow with unipolar 0 5 mm gauge stainless steel electrodes The stimuli currents consisted of pulses with 2 msec duration frequency 40—80 imp/sec and intensity 1—4 V Vasodilatation was also elicited by stimulating the lumbar sympathetic chain with a bipolar silver electrode at L4—L5 level The chain was reached using a retroperitoneal approach Stimulation was performed after blocking the vasoconstrictor nervous response usually with dihydroergotamine (Orstanorm®) 0 1 mg i a but sometimes by giving guanethidine (Ismeline®) 2 mg/kg i v to dogs pretreated with reserpine (Serpasil®) 1—2 mg/kg i m The stimuli

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Vasodilator nerve stimulation and acetylcholine infusion caused comparable increases in disappearance rate during the blood flow increase When the blood flow was back to resting levels a decrease in disappearance rate below control values was seen The increase in disappearance rate during sympathetic nervous vasodilatation was usually less than during metabolic vasodilatation and after metabolic vasodilatation no decrease was observed in the disappearance rate The results support the hypothesis that during metabolic vasodilatation precapillary sphincters are opened up leading to an increased capillary surface area while the vasodilator nerves do not directly influence the exchange surface area

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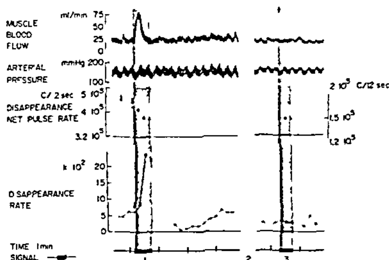


Fig 1 Dog 24 kg Pentobarbital M gastrocnemius tibialis 146 g Effect of stimulation of lumbar sympathetic chain Dihydroergotamine 0 1 mg i.a had been given previously to block the vasoconstrictor nervous response

1 Stimulation with 6 V 2 msec 5 imp/sec Isotope I^{125}
2 Atropine 0 1 mg i a
3 Stimulation with 6 V 2 msec 5 imp/sec Isotope Xe^{133}

Note that the disappearance rate is illustrated in two ways both as a semilogarithmic plot of the net pulse rate of the depot against time and below that as k values taken from the slopes between two consecutive readings of the same scaler The k value at the arrow was derived from the slope between the two points in the net pulse rate curve indicated by arrows

regulary after pronounced cholinergic vasodilatations and was observed with both tracers but most clearly using I^{125} Atropine 0 1 mg i a abolished the effects of stimulation indicating that sympathetic cholinergic vasodilator fibres had been stimulated (Fig 1 3)

Graded effects on the blood flow and the disappearance rate of Xe^{133} were obtained following vasodilator nerve stimulation (Fig 2) Similar response was obtained with I^{125} and no difference in this respect was observed between the two isotopes

Maximal vasodilatations due to sympathetic chain stimulation were produced with a duration of 1—10 msec It was sometimes possible to obtain effects though very minute ones using as low a duration as 0 1 msec but below that value no effects were obtained Increasing vasodilatory effects were produced by increasing the frequencies from 0 1 imp/sec up to 10 imp/sec The effective intensities varied from experiment to experiment but usually a threshold effect was obtained with 3—4 V Before the adrenergic blockade vasoconstrictor nervous responses were elicited by sympathetic chain stimulation In all experiments lower intensities and shorter durations were needed to produce vasoconstriction than to produce vasodilatation. Vasoconstrictions were produced by stimulating with 1 5—3 V and using durat

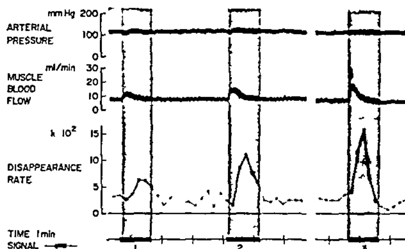


Fig 2 Dog 9 5 kg Pentobarbital M gastrocnemius 47 g Effect of stimulation of lumba sympathetic chain Dihydroergotamine 0 2 mg i a had been given previously to block th vasoconstrictor nervous response Isotope Xe^{133}

1 Stimulation with 8 V 2 msec 2 5 imp/sec
2 Stimulation with 8 V 2 msec 6 imp/sec
3 Stimulation with 8 V 2 msec 10 imp/sec

In this experiment atropine 0 1 mg i a abolished the effects on the blood flow and di appearance rate caused by stimulation

0 01—0 05 msec The finding of different stimulus thresholds for the vasomotor nervous effects is in agreement with Folkow Johansson and Öberg (1958) who obtained similar results in cat

Stimulation of hypothalamic vasodilator nervous pathways was performed in 10 dogs The effect following stimulation was more or less the same as that observed upon sympathetic chain stimulation the muscle blood flow and the disappearance rate were both increased (see Fig 3 1)

b *Comparison between the effects in different skeletal muscles* The disappearance rate from different muscles including the gastrocnemius was measured upon stimulation of hypothalamic vasodilator nervous pathways In four dogs *M masseter* was studied and in two of these the disappearance rate from *M splenius* and *M latissimus dorsi* was also followed In these experiments it was not possible for technical reasons to measure the blood flow to the muscle but the recording of the femoral blood flow served as a control that vasodilator nervous pathways were activated by the stimulation

Upon hypothalamic stimulation the disappearance rate was increased from the splenius and latissimus dorsi muscles but not from the masseter muscle Fig 3 illustrates one experiment with hypothalamic stimulation In Fig 3 1 stimulation gave rise to an increase in femoral blood flow The arterial pressure was unchanged The disappearance rate of I^{125} in the gastrocnemius muscle increased 3—4 fold In Fig 3 2 the effect of hypothalamic stimulation on the disappearance rate of Xe^{133} in the masseter muscle is shown The femoral blood flow was measured to test that

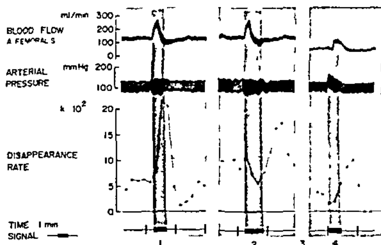


Fig 3 Dog 13 kg Morphine Chloralose Effect of stimulation of vasodilator pathways in the hypothalamus

1 Stimulation with 1 8 V 2 msec 80 imp/sec
[illegible]
2 [illegible]
3 [illegible]
4 [illegible]
[illegible] *gastrocnemius*

Note the difference in response between *M masseter* and *M gastrocnemius* before atropine

vasodilator nervous pathways were activated During stimulation of vasodilator pathways, indicated by the femoral blood flow increase, the disappearance rate of Xe^{133} in the masseter muscle was not increased but rather slightly decreased Upon cessation of the stimulation the disappearance rate was enhanced above control level The findings suggest that vasoconstriction was produced in the masseter muscle during stimulation of the hypothalamic vasodilator outflow The increased disappearance rate seen after the stimulation was probably due to a compensatory vasodilatation following the vasoconstriction

Atropine, 0 3 mg i a, (Fig 3 3) reduced the increase in femoral blood flow and disappearance rate produced by stimulation indicating that cholinergic vasodilator fibres had been activated (Fig 3 4) After atropine the stimulation elicited a rise in arterial pressure probably leading to baroreceptor activation and reflex inhibition of vasoconstrictor nervous tone This was indicated by the delayed increase in both femoral blood flow and disappearance rate from the gastrocnemius muscle which was observed following stimulation

Comparison between different types of vasodilatation

We compared the disappearance rate of I^{125} (6 experiments) and Xe^{133} (2 experiments) following sympathetic cholinergic vasodilatation with vasodilatations pro-

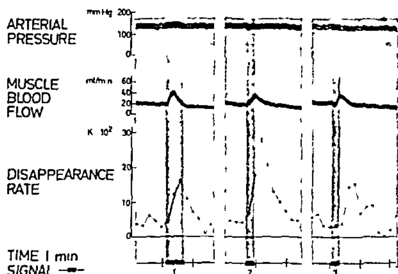


Fig 4 Dog 15 5 kg Pentobarbital M gastrocnemius-tibialis 105 g Comparison between different types of vasodilatation Dihydroergotamine 0 08 mg i a had been given previously to block the vasoconstrictor nervous response Isotope I^{125}

1 Lumbar sympathetic chain stimulation 8 V 1 5 msec 5 imp/sec
2 Motor nerve stimulation 5 V 0 01 msec 4 imp/sec
3 Acetylcholine infusion (0 3 µg/min i a)

Note the different effects on the disappearance rate in contrast to the similar blood flow increases caused by the different vasodilatory mechanisms

duced by motor nerve stimulation or acetylcholine infusion In Fig 4 one such experiment is illustrated The increases in blood flow obtained by the different procedures were about the same while the effects produced on the disappearance rate differed A four-fold increase in disappearance rate was observed following sympathetic nerve stimulation and on acetylcholine infusion By contrast the disappearance rate increased about seven times due to motor nerve stimulation Furthermore, after the sympathetic nervous and acetylcholine induced dilatations the disappearance rate decreased below resting values while this negative after-effect was not observed following metabolic vasodilatation

The results from the comparative study are collected in Table I The data indicate that the effect on the disappearance rate following sympathetic cholinergic vasodilatation was less than that after motor nerve stimulation In six out of seven experiments the mean value of the disappearance-flow ratio during metabolic vasodilatation exceeded that upon sympathetic vasodilator nerve stimulation and in one they were equal On the other hand no systematic difference was observed between the effects obtained during acetylcholine and sympathetic vasodilatation Different disappearance-flow ratio values were obtained during different parts of the wash-out curve indicated in the table by the wide ranges seen in response to the same type of vasodilatation. As a consequence of this the order in which the different types of dilatation were produced was randomized to avoid systematic errors

TABLE I Percentage increase in disappearance rate/percentage increase in blood flow (disappearance flow ratio) after sympathetic vasodilator nerve stimulation acetylcholine infusion and motor nerve stimulation Means and ranges were determined *using logarithms of the percentage increase values*
n number of stimulations or infusions

Comparison between disappearance/flow ratios on different types of vasodilatation							
		Symp nerve stim		Acetylcholine		Motor nerve stim	
Dog no	Isotope	n	Mean (range)	n	Mean (range)	n	Mean (range)
9	I^{125}	3	3 2 (1 1–12)	3	3 2 (2 8–3 9)	1	11 (–)
12	„	11	4 7 (2 7–11)	9	5 0 (2 0–10)	5	9 6 (5 0–19)
15	„	6	0 55 (0 27–0 81)	—	—	3	2 0 (1 6–2 3)
16	„	11	2 1 (0 74–3 9)	9	5 8 (4 2–8 3)	3	5 9 (4 5–8 5)
19	„	15	4 3 (2 1–9 1)	7	2 6 (1 7–6 2)	4	4 6 (1 4–8 3)
26	„	7	3 0 (1 5–7 8)	6	10 (6 5–29)	—	—
8	Xe^{133}	7	1 3 (1 0–1 9)	3	2 1 (1 3–3 5)	3	1 3 (1 0–1 7)
19	„	3	4 6 (2 8–7 6)	2	3 8 (2 6–5 5)	2	9 3 (9 3–9 3)

Discussion

In the present study the disappearance rate of a watersoluble (I^{125}) and a lipid-soluble (Xe^{133}) tracer was accelerated in the gastrocnemius muscle of the dog during sympathetic cholinergic vasodilatation, indicating an enhanced tissue blood exchange Barlow and Walder (1965) have reported similar results measuring the disappearance of Na^{24} from the gastrocnemius and soleus muscles of the cat.

Hyman *et al* (1959) did not find any increase in the disappearance rate of I^{131} in cat skeletal muscle following stimulation of hypothalamic vasodilator pathways One explanation for this might be that they used a device for radioisotope measurements which was incapable of detecting rapid changes Furthermore, Hyman *et al* (1959) used an isolated, heparinized muscle preparation and measured blood flow with a drop recorder Using the same type of preparation and measuring blood flow with a drop recorder we obtained in a few experiments increased blood flow by vasodilator nerve stimulation but failed to observe any change in the I^{125} disap-

pearance rate In these experiments the disappearance rate was exceptionally slow during resting conditions The findings suggest that in experiments where extra corporeal circulation is used the transcapillary exchange might be disturbed due to rheological changes e g intravascular aggregation of erythrocytes

As seen in Table I the disappearance flow ratio exceeded 1 in 21 out of 22 trials showing that the disappearance rate increased more than the blood flow during the different types of vasodilatation studied Many investigators (Lassen Lindbjerg and Dahn 1965, Kjellmer *et al* 1967, Tonnesen and Sejrsen 1967, Sejrsen and Tonnesen 1968) have found a consistent correlation between the disappearance rate of fat soluble tracers and the blood flow, a finding which seems to disagree with our results However these investigations differ from ours in many respects In some of them other methods to load the tissue with isotope, e g labelling the tissue by intra arterial infusion of the tracer, have been used Furthermore, the findings that changes in disappearance rate are similar to changes in blood flow has been made during the initial phase of the wash out curve (*e g* Kjellmer *et al* 1967) while we performed our study during the subsequent mono exponential part A further important point is that the analyses showing linear correlation between disappearance rate and total blood flow have been made during 'steady state' conditions and not during rapid changes of the blood flow as in our work (*e g* Sejrsen and Tonnesen 1968)

Several factors might have been responsible for producing the great increase in *disappearance rate observed during vasodilatation in our experiments For example* the rapid wash out of blood equilibrated with the tissue in respect to isotope content might contribute to give the initial high disappearance rate (*cf* Lassen 1964) Moreover an increase in the surface area available for tissue blood exchange during the vasodilatation would in itself lead to an increased disappearance rate This is illustrated by our finding (Bolme and Edwall to be published) that even when the blood flow was kept constant at resting level the disappearance rate of I^{125} and Xe^{133} from a muscular depot increased during motor nerve stimulation a procedure which has been shown to increase the exchange surface area (Krogh 1929 Renkin and Rosell 1962 Kjellmer 1964) That disappearance rate of water soluble material may increase in some situations without any change in blood flow has also been shown by Lundgren and Mellander (1967) These authors observed an increased tissue blood transfer of water soluble agents caused by increased bulk filtration or absorption without any concomitant change in total blood flow

The effect on the disappearance flow ratio following sympathetic vasodilatation was similar to that following acetylcholine infusion This observation is in agreement with the finding of Bolme and Novotny (1969 b) that sympathetic nerve stimulation and acetylcholine produces similar effects on the oxygen uptake in skeletal muscle of the dog In our opinion the effects observed after sympathetic vasodilator nerve stimulation and acetylcholine might be explained by an action mainly exerted on precapillary resistance vessels However Djojosugito *et al* (1968) reported a difference between the effect on CFC (capillary filtration coefficient) during acetylcholine infusion and that during sympathetic vasodilator nerve stimulation in the

cat the CFC increased during acetylcholine infusion but decreased during vasodilator nerve stimulation. This finding seems to be at variance to our results. The discrepancy might be explained by a species difference but also by the fact that we studied the initial effects while Djojosugito *et al.* (1968) studied the CFC effects during prolonged dilatations.

After the dilatations caused by acetylcholine and vasodilator nerve stimulation, when the blood flow was back to resting level, the disappearance rate was often decreased far below control values. This phenomenon may be due to precapillary sphincter closure caused by autoregulatory mechanisms. The increased transmural pressure would result in a myogenic constriction at the level of the precapillary sphincters and consequently the surface area available for exchange would be reduced (*cf.* Folkow and Öberg 1961, Rosell and Uvnäs 1962, Mellander, Öberg and Odelram 1964).

In nearly every case the disappearance flow ratio was higher during the metabolic than during the dilatation due to vasodilator nerve stimulation. Moreover, an increased disappearance was often seen after metabolic vasodilatation when the blood flow was back to normal. These results may be interpreted in accordance with previous findings that during metabolic vasodilatation precapillary sphincters are dilated thereby leading to an increased surface area for exchange (Krogh 1929, Renkin and Rosell 1962, Kjellmer 1964).

In some experiments we stimulated sympathetic vasodilator nervous pathways in the hypothalamus and measured the disappearance from different skeletal muscles including the gastrocnemius. During the stimulation the disappearance was increased in the gastrocnemius, the splenius and the latissimus dorsi muscles but not in the masseter muscle. Thus, the findings indicate that differences exist in the distribution of sympathetic vasodilator nerves to various muscles. Our results are supported by the findings made in a histochemical study (Bolme and Fuxe, to be published) that small arteries (30—100 μ) in the gastrocnemius muscle but not in the masseter muscle seem to be innervated by sympathetic cholinergic fibres. The results might indicate that only arteries in muscles used for movements of the extremities, e.g. during running, jumping etc., are innervated by sympathetic cholinergic vasodilator fibres. This assumption is in agreement with the recent findings in conscious dogs that the vasodilator nerves are activated as conditional reflex prior to exercise on a treadmill (Bolme and Novotny 1969 a).

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Acta physiol scand. 1970 78. 39—42

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Effect of Salicylate Treatment on Fetal and Maternal Prothrombin Time in the Mouse

By

MARGARETA ERIKSSON

Received 6 March 1969

Abstract

ERIKSSON, M *Effect of salicylate treatment on fetal and maternal prothrombin time in the mouse* Acta physiol scand 1970 78 39—42

In order to investigate earlier observed salicylate induced fetal hemorrhage sodium salicylate or dicumarol was given i m. to A/Jax mice on the 17th gestation day Prothrombin time was measured in the fetuses and the mother at autopsy at different times after injection A decrease in the prothrombin concentration was found in fetuses with salicylate induced hemorrhage after 4 8 and 12 hrs but not after 24 hrs Fetuses without hemorrhages and the mothers in the salicylate group had the same prothrombin time as those in the control groups Dicumarol lowered the prothrombin concentration in both the mother and fetuses but did not cause visible hemorrhage The possible correlation between salicylate induced hemorrhage and hypoprothrombinemia is briefly discussed

Hemorrhages have recently been induced in mouse fetuses by sodium salicylate treatment late in pregnancy (Eriksson in press) Salicylates are known to produce hemorrhage in adults and many underlying mechanisms have been suggested (Smith and Smith 1966) among them hypoprothrombinemia (Meyer and Howard 1943, Quick and Clesceri 1960)

A prolonged prothrombin time has been observed in fetuses compared to that in adults (Zilliacus *et al* 1964 Caruso and Petrakis 1966) The present investigation was undertaken to ascertain whether sodium salicylate administration to pregnant mice would further decrease the already low prothrombin concentration in the fetus Such a decrease might partly explain the development of salicylate induced fetal hemorrhage

Material and methods

Pregnant primiparous mice of the A/Jax strain—inbred in the laboratory (Larsson 1962)—to a total number of 52 were used They had been mated overnight and the day when vaginal plug was observed is denoted as zero day of pregnancy

They were injected i m with 0 1 ml of solution/20 g b w at 10 a m on the 17th gestation day (Table 1) The substances : sodium salicylate (100 mg/ml distilled wa

TABLE I Maternal and Fetal Prothrombin Time and Fetal Damage in the A/Jax Strain Treated

Substance given i.m. 0.1 ml/20 g	Gestation day injected	Time of sacrificing after inj. hours	Number of litters	Number of fetuses	Number of fetuses dead
Salicylate*	17	4	9	64	0
"	17	8	8	53	11
"	17	12	7	46	17
"	17	24	7	42	8
Dicumarol**	17	8	8	61	0
Untreated controls 17th gestation day			8	55	0
Untreated controls 18th gestation day			5	36	0

* Sodium salt of salicylic acid, 100 mg/ml distilled water

dicumarol[1] 10 mg/ml saline. Uninjected mice on the 17th and 18th gestation days served controls.

At 4, 8, 12 or 24 hrs after injection the animals were anesthetised with Nembutal® (1 mg/20 g) for blood sampling. Two 10-μl samples of blood were taken by capillary pipet from the tail of the mother. Immediately afterwards the fetuses were removed and exam for death and superficial hemorrhages (Table I). Early fetal resorption which had obviou occurred before treatment were not recorded. Not more than four fetuses from each l were taken for blood sampling. From each fetus a 10-μl sample was obtained from a deep c in the neck.

The 10-μl samples were blown into a tube with 1.5 μl of 3.8 % sodium citrate. The pr thrombin time was estimated in a two-stage procedure with freeze-dried reagents[2] as d scribed in detail by Noren and Blombäck (in preparation). To the ampoules with pr thrombin reagent containing an activated mixture of the factors in the first two steps coagulation except for prothrombin 1 ml of distilled water was added and warmed for 3 m in a water bath at 37° C. The solution was added to the blood sample and warmed f another 3 min, after which 0.2 ml was mixed with 0.2 ml of 0.4 % samples of fibrinog solution in Tris buffer pH 7.4. The freeze-dried fibrinogen was reconstituted with water a warmed in the water bath for 4 min before use. The coagulation time was recorded in secon and the mean values and standard deviation are given in Table I.

Results

The prolonged prothrombin time in mouse fetuses compared to that in adu found by Carus and Petrakis (1966) was verified (Table I). Moreover injectio of sodium salicylate to pregnant mice on the 17th gestation day produced after 4 and 12 hrs a further decrease in prothrombin concentration in fetuses with macr scopicaly visible superficial hemorrhage. 24 hrs after injection of sodium salicyla no decrease in prothrombin concentration of the fetus could be detected desp

[1] Waran®, Sodium 3-(α-acetonylbenzyl)-4-hydroxycumarin
[2] All reagents were received from the Department of Blood Coagulation Research, Karolinsk Institutet.

with Sodium Salicylate and Dicumarol

Number of living fetuses with superficial hemorrhage	Prothrombin time Mean and SD								
	Mothers			Fetuses unaffected			Fetuses with superficial hemorrhages		
	no	sec		no	sec		no	sec	
33	9	45	3	15	164	8	9	319	45
25	7	42	1	8	148	13	12	343	40
9	6	40	3	11	183	12	8	305	40
10	7	41	2	11	130	5	6	139	9
0	7	95	3	21	295	13	0	—	
0	7	43	2	19	167	7	0	—	
0	3	40	2	15	136	8	0	—	

** Waran®, Sodium 3 (α acetonylbenzyl) 4-hydroxicumarin, 10 mg/ml saline

visible hemorrhage Macroscopically unaffected fetuses had the same prothrombin time as fetuses of uninjected control mothers Injection of sodium salicylate did not affect the prothrombin time in the mother

Dicumarol injection resulted in a prolonged prothrombin time and 8 hrs on the 17th gestation day, in both mother and fetus Despite the decreased prothrombin time no visible hemorrhage was observed

Discussion

The prolonged prothrombin time shown in the mouse fetuses with salicylate induced hemorrhages might be one of the causes of *this type of fetal damage This theory is* supported by the fact that no lowering of the prothrombin concentration was observed in the unaffected fetuses, nor in the mothers which did not show hemorrhages Salicylate treatment in adults has been shown to cause a lowering of the prothrombin *concentration in some cases associated* with hemorrhages (Wising *1952*)

The fact that dicumarol treatment resulted in a lowering of prothrombin concentration in the mothers, as well as in the fetuses, without giving rise to any hemorrhages indicates that a low prothrombin concentration is not the only causative factor It is interesting to note that the prothrombin concentration in the fetuses was as low as in the fetuses with hemorrhage in the salicylate treated group

It can be surmised that the salicylate *induced fetal hemorrhages are caused* by a combination of different factors one of them being hypoprothrombinemia Other earlier suggested factors are capillary fragility trombocytopenia and hypoadhesivity of platelets, as reviewed by Smith and Smith (1966) Of these factors, capillary fragility is the most likely one in this case as the salicylates also have the possibility

of depressing the synthesis of acid mucopolysaccharides which are an important constituent of the vessel wall (Boström and Månsson 1955, Jacobson *et al.* 1964). This combination of mechanisms has also been suggested for salicylate hemorrhage in humans (Frick 1956). It is interesting to note that after 24 hrs the prothrombin time is restored to normal and the only sign of salicylate treatment is unresorbed hematomas.

Another question is why salicylate treatment causes hypoprothrombinemia in the fetus but not in the mother. Prothrombin is synthesized by the liver (Barnhart 1965) and any damage to the liver would therefore affect the prothrombin concentration. It can be speculated that the immature fetal liver might be more sensitive to any liver-damaging substance, *e.g.* salicylate. Salicylates have been shown to cause liver damage in adults and children (Manso *et al.* 1956). Recently salicylate has also been demonstrated to induce subcapsular liver hemorrhage in fetal mice (Eriksson in press). The salicylate-induced hypoprothrombinemia could therefore be interpreted as a sign of liver damage rather than mediated through a specific action on the synthesis of prothrombin.

I take this opportunity for expressing my sincere gratitude to Drs. B. Blombäck and K. S. Larsson for valuable advice and help during the investigation. I also wish to thank Mrs. Anne-Marie Johansson for skilful technical assistance and Mrs. Tollie Palm for excellent secretarial help.

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Acta physiol scand. 1970. 78. 43—51

From the Psychiatric Research Center, Ulleråker Hospital University of Uppsala and the Department of Physiology Karolinska Institutet, Stockholm Sweden

A Method for the Simultaneous Determination of 5-Hydroxy-3-Indole-Acetic Acid (5-HIAA) and 5-Hydroxytryptamine (5-HT) in Brain Tissue and Cerebrospinal Fluid

By

JOHN JONSSON and TOMMY LEWANDER

Received 7 March 1969

Abstract

JONSSON, J and T LEWANDER *A method for the simultaneous determination of 5 hydroxy 3 indole acetic acid (5-HIAA) and 5 hydroxytryptamine in brain tissue and cerebrospinal fluid* Acta physiol scand 1970 *78* 43—51

A *method is described for the isolation of* 5 HIAA *in brain tissue extracts or cerebrospinal* fluid by use of a Sephadex G 10 column The eluate is taken for fluorimetric determination of 5 HIAA in 3 M hydrochloric acid Tissue blanks are obtained by destruction of 5 HIAA with potassium ferricyanide plus UV irradiation or with hydrogen peroxide Recovery of 5 HIAA from brain extracts was above 90 % The *sensitivity of the method* allows quantitative

In the present paper a method is described for the isolation of 5 HIAA in tissue extracts and cerebrospinal fluid (CSF) by adsorption and/or ion exchange chromatography on small Sephadex G 10 columns The compound is concentrated in a small eluate volume and determined fluorimetrically This procedure constitutes a modification of the method of Contractor (1966) for the determination of 5 HIAA in urine The present method seems to have advantages over previous solvent extraction procedures for isolation of 5 HIAA (Roos 1962 Giacalone and Valzelli 1966) with regard to sensitivity, recovery and reproducibility The combination of the present 5 HIAA method with the methods of Bertler (1961) and Anden and Magnusson (1967) for determination of 5 HT could be used for the simultaneous determination of both compounds in a single rat brain

The normal contents of 5 HIAA and 5 HT whole brain and in certain parts of the rat brain have been determined by this procedure Data on rat brain 5 HIAA

of depressing the synthesis of acid mucopolysaccharides which are an important constituent of the vessel wall (Bostrom and Månsson 1955, Jacobson *et al* 1964) This combination of mechanisms has also been suggested for salicylate hemorrhage in humans (Frick 1956) It is interesting to note that after 24 hrs the prothrombin time is restored to normal, and the only sign of salicylate treatment is unresorbed hematomas

Another question is why salicylate treatment causes hypoprothrombinemia in the fetus, but not in the mother Prothrombin is synthesized by the liver (Barnhart 1965) and any damage to the liver would therefore affect the prothrombin concentration It can be speculated that the immature fetal liver might be more sensitive to any liver damaging substance *e g* salicylate Salicylates have been shown to cause liver damage in adults and children (Manso *et al* 1956) Recently, salicylate has also been demonstrated to induce subcapsular liver hemorrhage in fetal mice (Eriksson in press) The salicylate induced hypoprothrombinemia could therefore be interpreted as a sign of liver damage, rather than mediated through a specific action on the synthesis of prothrombin

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[illegible] recovery of [illegible]

In the present paper a method is described for the isolation of 5 HIAA in tissue extracts and cerebrospinal fluid (CSF) by adsorption and/or ion exchange chromatography on small Sephadex G-10 columns The compound is concentrated in a small eluate volume and determined fluorimetrically This procedure constitutes a modification of the method of Contractor (1966) for the determination of 5 HIAA in urine The present method seems to have advantages over previous solvent extraction procedures for isolation of 5-HIAA (Roos 1962 Giacalone and Valzelli 1966) with regard to sensitivity recovery and reproducibility The combination of the present 5-HIAA method with the methods of Bertler (1961) and Andén and Magnusson (1967) for determination of 5 HT could be used for the simultaneous determination of both compounds in a single rat brain

The normal contents of 5-HIAA and 5 HT whole brain and in [illegible] the rat brain have been determined by this procedure Data on rat

and 5 HT levels after malamide, α propyldopacetamide (H22/54), p chlorophenyl alanine and probenecid are presented

Furthermore, cerebrospinal fluid (CSF) levels of 5-HIAA in 7 human subjects were determined in parallel with the present Sephadex method and the method of Ashcroft and Sharman (1960) and the results are compared

Material and methods

Sephadex G 10 was obtained from Pharmacia Fine Chemicals (Uppsala Sweden) and 5 hydroxyindole acetic acid from Sigma Chem Co Bidistilled (quartz glass) water and *pro analysi* reagents were used throughout the study

Samples of 5 HIAA carboxyl ^{14}C (1 88 mC/mmole, purity >95 % according to the present method) and 5 HT 2 ^{14}C (10 4 mC/mmole purity >96 % according to the ion exchange chromatographic method of Bertler (1961) were obtained from New England Nuclear Corp Radioactivity was measured by liquid scintillation (Packard Tri Carb) using a 7 3 toluene absolute alcohol solution containing 4 g of PPO and 100 mg of POPOP per liter of toluene as scintillation mixture Quenching was monitored by the external standard technique

Determination of 5 HIAA in rat brain

A *Extraction of 5 HIAA from brain tissue*

Single brains from Sprague Dawley rats (180—220 g b w) were homogenized (Ultra Turrax) in 6 ml of 0 1 M HCl containing 0 5 % ascorbic acid The epiphysis had been removed Proteins were precipitated by addition of 1 0 ml 10 % zinc sulphate and 0 6 ml 1 M NaOH The homogenates were centrifuged at 10 000 × g for 10 min in a refrigerated centrifuge Re extraction of the tissue was performed in 3 0 ml of 0 1 M HCl and the proteins were pre cipitated with 0 5 ml zinc sulphate plus 0 3 ml NaOH After a second centrifugation 0 1 ml of 10 % EDTA (di sodium salt of ethylene diamine tetraacetic acid) was added to the pooled supernatants which were filtered through paper or glass wool Of the filtered extract 9 0 ml was taken for analysis and the pH of this aliquot was adjusted to 1 5—2 0 by addition of 1 M HCl

B *Column procedure*

Glass columns (I D 5 mm) were packed with 0 5 [illegible] The Sephadex beads had swelled in 0 1 M HCl for at [illegible] washed with 10 ml of 0 1 M HCl containing 0 1 [illegible] the sample When the extract had passed through [illegible] 0 1 M HCl containing 0 1 % ascorbic acid and 1 5 ml of water Elution of 5 HIAA was performed with 2 5 ml 0 02 M ammonium hydroxide

C *Fluorimetric determination*

The eluate was divided into three portions which were used for sample internal standard and tissue blank These mixtures were read against a 5 HIAA standard (set to 50 F U = fluorescence units) in an Aminco Bowman spectrophotofluorimeter at 300/540 nm (excitation/emission wavelengths uncorrected instrumental values) The procedure is illustrated by Table I Silica test tubes were used Destruction of 5 HIAA in the tissue blank was obtained by addition of potassium ferricyanide and irradiation of the tube for 10 min in UV light (original Hanau UV tube NN 30/89) Surplus potassium ferricyanide was removed by addi tion of ascorbic acid

Determination of 5 HT in rat brain

For the simultaneous determination of 5 HIAA and 5 hydroxytryptamine (5 HT) in the same rat brain the effluent and washings from the Sephadex columns were adjusted to pH 5—6 with 1 M NaOH and the protein precipitate was removed by centrifugation at 10 000 × g for 10 min Isolation of 5 HT was made on Amberlite XE 64 ion exchange columns according to Bertler (1961) and the fluorimetric determination was made as described by Anden and Magnusson (1967)

Determination of 5 HIAA in human CSF

Human CSF was obtained by lumbar puncture The sample was immediately frozen and stored at −30° C for a maximum of one week before determination of 5 HIAA To a 5 ml aliquot

TABLE I Composition of samples prepared for the fluorimetric determination of 5 HIAA from rat brain extracts in eluates from Sephadex G 10 columns (500 mg) Figures represent ml of the respective reagents or solutions

	Sample	Internal standard	Tissue blank	Standard	Reagent blank
Water	0 1	—	0 1	—	0 1
Ascorbic acid 1 %	0 1	0 1	—	0 1	0 1
$K_4Fe(CN)_6$ 0 025 %	0 1	0 1	0 1	0 1	0 1
Eluate	0 8	0 8	0 8	—	—
NH_4OH 0 02 N	—	—	—	0 8	0 8
5 HIAA 5 μg/ml	—	0 1	—	0 1	—
HCl conc	0 5	0 5	0 5	0 5	0 5
Irradiation i UV light 10 min					
Ascorbic acid 1 %	—	—	0 1	—	—

of the CSF was added 0 17 ml of 70 % perchloric acid for precipitation of proteins The pH was adjusted to 1 5—2 0 by addition of 2 M KOH and the precipitate was spun down at 10 000 × g for 10 min The 5 HIAA was isolated on a Sephadex G 10 column as described above Aliquots of 0 8 ml of the eluate were taken for preparation of the samples internal standards and tissue blanks in silica test tubes as shown in Table II These mixtures were read against a standard containing 0 20 μg 5 HIAA and a reagent blank

Results and comments

1 *Extraction of 5 HIAA from brain tissue*

In the present study extraction of 5 HIAA from brain tissue with 0 1 M HCl and deproteinization with zinc sulphate and sodium hydroxide was preferred Other extraction media, notably 0 4 M perchloric acid, 5 % trichloracetic acid and 10 % metaphosphoric acid, was found to solubilize material from the brain tissue that interfered with the fluorimetric measurement of 5 HIAA by causing high tissue blank readings

TABLE II Composition of samples prepared for the fluorimetric determination of 5 HIAA from cerebrospinal fluid in eluates from Sephadex G 10 columns Figures represent ml of the respective reagents or solutions

	Sample	Internal standard	Tissue blank	Standard	Reagent blank
Water	0 2	—	0 2	—	0 2
Cystein, 0 1 %	0 1	0 1	—	0 1	0 1
H_2O_2 1 100	—	—	0 1	—	
Eluate	0 8	0 8	0 8	—	
NH_4OH, 0 02 N	—	—	—	0 8	0 8
5 HIAA 1 μg/ml	—	0 2	—	0 2	
HCl conc	0 5	0 5	0 5	0 5	0 5

TABLE III Recovery of ^{14}C-labeled 5 HIAA and 5 HT to the extraction medium before homogenization of the brain tissue (whole brain) The amounts of radioactivity in the supernatants after centrifugation of the homogenates are expressed as the percentages ± S D of the added amounts
n = number of observations
n m = not measured

Compound added	*Mode of extraction*	*Extr 1*	*Extr 2*	*Extr 3*	*Total* extractable
5 HIAA ^{14}C	HCl, $ZnSO_4$ NaOH (n = 5)	61±2 1	17±0 4	~1	78±1 0
0 5 μg =	$HClO_4$ (n = 4)	62±1 3	16±0	n m	78±1 6
10 000 dpm					
5 HT ^{14}C	HCl $ZnSO_4$ NaOH (n = 3)	67±3 0	14 ±10	n m	81±3 8
0 2 μg =	$HClO_4$ (n = 4)	79±0 6	n m	n m	79±0 6
22 000 dpm					

In Table III the extraction efficiencies for HCl and perchloric acid respectively are compared It was found that only about 80 % of labelled 5 HIAA or 5 HT added to brain tissue homogenates was recovered even after reextraction of the tissue In similar experiments the recovery of noradrenaline was 100±2 5 % (mean ±S D), dopamine 88±2 6 % and normetanephrine 95±4 0 % after a single extraction with perchloric acid These results favour the view that the 5 hydroxyindoles may be bound to tissue constituents

A single extraction of rabbit brain tissue with perchloric acid yielded about 74 % of the total extractable 5-HT (Anden and Magnusson 1967) This figure is in agreement with that of the present study (79 %, Table III)

2 *Recovery of 5 HIAA and 5-HT from brain tissue extracts*

To tissue extracts from single rat brains were added 0 5 μg of 5 HIAA Recoveries with cold 5 HIAA were 91 ± 3 7 % (mean ± S D) and with labeled 5 HIAA 96± 2 0 %

After addition of 5 HT ^{14}C (~0 2 μg) to rat brain extracts, 92±6 0 % (mean ±S D) of the radioactivity was found in the effluent and washings from the Sephadex G 10 columns The recovery of 5 HT-^{14}C over the Amberlite XE-64 column was 92 ± 2 6 % (mean ± S D) The recovery of extractable 5 HT over both column steps was thus about 85 %

3 *Reproducibility of the method*

Extracts from 10 single rat brains were pooled and divided into 10 equal samples that were run simultaneously *The total amount of 5 HIAA per sample* was 0 28± 0 01 (M±S D) μg with a range of 0 27—0 29 μg Determination of 5 HT was performed in the same 10 samples and the total amount of the amine per sample was 0 45±0 02 (mean ± S D) μg ranging from 0 43 to 0 48 μg

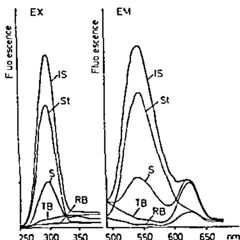


Fig 1

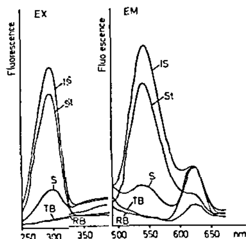


Fig 2

Fig 1 Excitation (EX) and emission (EM) spectra of a rat brain sample (Sephadex G 10 eluate) recorded at 545 nm and 295 nm respectively St=0 5 µg for 5 HIAA S= sample IS=internal standard TB=tissue blank RB=reagent blank

Fig 2 Excitation (EX) and emission (EM) spectra of a CSF sample (Sephadex G 10 eluate) recorded at 545 nm and 295 nm respectively St=0 2 µg of 5-HIAA For explanation of abbreviations see text to fig 1

4 *Stability of 5 HIAA in rat brain extracts*

The extracts from 9 rat brains were pooled and measured for 5 HIAA in triplicate a) immediaely, b) at day 3 and c) at day 7 after the homogenization The tissue extract was stored at pH 4 5 and at −30° C During the first 3 days there was a reduction of 5 HIAA of about 3 % and after 1 week a reduction of 16 % of the initial amount of 5 HIAA was observed

5 *Fluorimetry of 5 HIAA*

The excitation and emission spectra for 5 HIAA in a rat brain sample are shown in Fig 1 For the preparation of tissue blanks destruction of 5 HIAA in the eluate was performed by addition of Fe^{3} ions plus irradiation with ultraviolet light as described by Anden and Magnusson (1967) for the fluorimetric determination of 5 HT Destruction of 5 HIAA by addition of hydrogen peroxide to the blank was equally effective This technique was used for CSF samples Excitation and emission spectra from a CSF sample are shown in Fig 2

6 *Specificity of the 5 HIAA determination*

A complete separation of 5 HIAA from its precursors 5 hydroxytryptophan and 5 HT is performed with the Sephadex G 10 column Of other substances that can interfere with the present method 5 hydroxytryptophol and N acetyl 5 methoxy tryptamine (melatonin) were only partially removed by the Sephadex G 10 column step Their fluorescence spectra were found to be identical to those of 5 HIAA (*cf.*

TABLE IV Effect of drugs on the levels of 5 HIAA and 5 HT in the rat brain Values represent means ± s e m Number of observations within brackets

Treatment	Dose mg/kg i p	Time after injection hrs	5 HIAA µg/g	%	5 HT µg/g	%
Saline	—	1	0 27 ± 0 01(15)	100	0 46 ± 0 01(14)	100
Nialamide	250	1	0 16 ± 0 01(10)[1]	59	0 65 ± 0 01(10)[1]	141
Nialamide	250	3	0 05 ± 0 01(9)[1]	18	0 90 ± 0 05(10)[1]	195
Saline	—	3	0 27 ± 0 03(6)	100	0 39 ± 0 03(6)	100
p Cl phenylalanine	500	3	0 18 ± 0 03(6)[3]	67	0 31 ± 0 03(6)[3]	80
p Cl phenylalanine	500	6	0 10 ± 0 02(6)[1]	37	0 22 ± 0 03(6)[2]	56
Saline	—	4	0 27 ± 0 02(10)	100	0 37 ± 0 02(10)	100
α propyldopacetamide (H22/54)	400	4	0 09 ± 0 02(10)[1]	33	0 26 ± 0 02(9)[2]	70
Saline	—	1	0 27 ± 0 01(15)	100	0 46 ± 0 01(14)	100
Probenecid	150	1 5	0 74 ± 0 05(12)[1]	274	0 49 ± 0 01(12)	106

[1] Drug versus saline $p < 0\ 001$

[2] Drug versus saline $p < 0\ 01$

[3] Drug versus saline not statistically significant

Udenfriend 1959) but the intensity of the fluorescence is weaker one to three fifths of that of 5 HIAA However, thin layer chromatography of the Sephadex G 10 eluates after purification of brain extracts or CSF revealed only one spot than was isochromatographic with 5 HIAA

Silica gel plates (0 25 mm) were run in ethylacetate acetic acid (180/0 5) and the spots were visualized by spraying with van Urk s reagent (0 5 g p dimethylaminobenzaldehyde in 25 ml of concentrated hydrochloric acid and 25 ml of 96 % ethanol) The Rf values in this system was for 5 HIAA (blue violet) 0 33 for 5 hydroxy tryptophol (violet) 0 43 and for N acetyl 5 methoxytryptamine (blue) 0 13

Thus there is no indication for the presence of 5 hydroxytryptophol or melatonin in the final sample and if present their contribution to the fluorescence readings appear to be insignificant

7 *Sensitivity*

The present method allows quantitative determination of 5 HIAA in brain extracts or aliquots of CSF down to about 50 ng This amount of 5 HIAA will give fluorescence readings of 2—3 times the tissue blank value

8 *Contents of 5 HIAA and 5 HT in rat brain*

According to the present method the brain content of extractable 5 HIAA in untreated male rats (180—210 g b w) measured 0 28 ± 0 01 µg/g wet tissue weight

(mean ± S E M n = 31 uncorrected for recovery) In the same rat brains the content of 5 HT was 0 39 ± 0 01 μg/g There was no statistically significant difference in brain 5 HIAA content between rats sacrificed by a blow on the head (0 30 ± 0 02 μg/g n = 10) or by chloroform narcosis (0 27 ± 0 01 μg/g n = 21)

The values of 5 HIAA in the rat brain as estimated by the present method is in agreement with those found by other procedures (Ashcroft Eccleston and Crawford 1965 Juorio and Vogt 1965 Pletscher *et al* 1964 Giacalone and Valzelli 1966)

For determination of 5 HIAA in different regions of the rat brain three brain parts had to be pooled The level of 5 HIAA in hypothalamus + thalamus was 0 61 ± 0 07 μg/g (mean ± s e m n = 3) in the brain stem (mesencephalon + pons + medulla oblongata) 0 56 ± 0 13 μg/g and in telencephalon (the remaining parts of the brain except cerebellum) 0 25 ± 0 01 μg/g Thus the distribution of 5 HIAA within the rat brain was found to be similar to that in the rabbit brain (Roos 1962)

9 *Effect of drugs on the levels of 5 HIAA and 5 HT in the rat brain*

For evaluation of the usefulness of the combined methods for determination of 5 HIAA and 5 HT the two compounds were simultaneously determined in single rat brains after administration of drugs which actions on central 5 HT neurones are known

After inhibition of monoamine oxidase with nialamide there was a decrease in brain 5 HIAA and a simultaneous increase in the 5 HT level as has been observed after other monoamine oxidase inhibitors in rabbits and rats (Roos 1962 Tozer Neff and Brodie 1966)

Both 5 HIAA and 5 HT decreased after administration of the inhibitors of the tryptophane hydroxylase step in the synthesis of 5 HT *p* chlorophenylalanine (Koe and Weissman 1966) and *a* propyl-dopacetamide (H22/54 Carlsson Corrodi and Waldeck 1963) The 5 HIAA level was found to decrease more rapidly than the 5 HT level after both synthesis inhibitors in the present experiments

After probenecid which drug inhibits the efflux of 5 HIAA from the brain tissue into the circulation there was a marked increase in the 5 HIAA level while brain 5 HT was unchanged in agreement with previous reports (Werdinius 1967 Neff Tozer and Brodie 1967)

As shown in the present experiments the pattern of changes in brain 5 HIAA and 5 HT caused by a drug may give valuable information on its mechanism of action on the 5 HT neurones

10 *Cerebrospinal fluid*

The use of perchloric acid for precipitation of CSF proteins was preferred to the use of $ZnSO_4$ and NaOH since the precipitate was more easily spun down by centrifugation Before precipitation of proteins 0 20 μg of cold 5 HIAA or about 0 5 μg of ^{14}C labeled 5 HIAA was added to 3 and 5 aliquots of CSF (5 ml each) respectively The recovery was 88 ± 2 3 % (mean ± S D)

Lumbar CSF samples from 7 men (42—55 years of age) w and analyzed

TABLE V Level of 5 HIAA (ng/ml) in human CSF The sample from each of the 7 persons was divided and determined by the two methods indicated in the table The values obtained by the Sephadex method are corrected for a recovery of 88 %

Person nr	Direct fluorimetry Ashcroft and Sharman 1960	The Sephadex method	Difference
1	0 032 μg/ml	0 037 μg/ml	+0 005 μg ml
2	0 033 ,,	0 033 ,,	—0 000 ,,
3	0 026 ,,	0 023 ,,	—0 003
4	0 038 ,,	0 037 ,,	—0 001
5	0 031 ,,	0 026 ,,	—0 005
6	0 037 ,,	0 035 ,,	—0 002 ,,
7	0 035	0 035 ,,	—0 000 ,,
mean ± S E M	0 033 ± 0 001	0 032 ± 0 001	

according to Ashcroft and Sharman (1960) and the present method simultaneously The results are shown in Table V The agreement between the two methods is striking These results indicate that the more simple procedure of direct fluorimetry of CSF samples is satisfactory for routine determinations of 5 HIAA However under certain circumstances, *e g* drug treatments, tryptophan loading, the more specific and equally sensitive Sephadex method is advocated

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Adrenergic and Cholinergic Nerve Terminals in Skeletal Muscle Vessels[1]

By

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Abstract

BOLME, P and K FUXE *Adrenergic and cholinergic nerve terminals in skeletal muscle vessels* Acta physiol scand 1970 78 52—59

By the use of histochemical methods the enzyme acetylcholinesterase (AcChE) was visualized in nerve terminal appearing structures in the adventitia of small intramuscular arteries (30— [illegible] the muscle, adrenergic nerve terminals were histochemically visualized Adrenergic nerve terminals were seen in the same layer of the vessel wall as the cholinergic ones, i e in the adventitia surrounding the media Adrenergic nerve terminals were found to innervate both large and small arteries In muscle samples from monkey and human subjects no AcChE rich nerve terminals were observed around the vessels However, adrenergic nerve terminals were found in these species with the same appearance as in cat and dog This finding supports previous physiological experiments indicating that skeletal muscle of monkey lack sympathetic cholinergic vasodilator innervation

It is generally believed that the sympathetic cholinergic vasodilator fibres innervate precapillary resistance vessels in skeletal muscle of cat and dog The evidence for this localization of the vasodilator nerve terminals is derived from physiological experiments (Folkow Mellander and Öberg 1961, Renkin and Rosell 1962 Rosell and Uvnäs 1962

Furthermore Folkow Öberg and Rubinstein (1964) speculated that the vasodilator nerves innervate predominantly the internal sheat of the muscle wall of the vessel, while the vasoconstrictor nerves innervate its external sheath

The present study is an attempt to elucidate the distribution and localization of the sympathetic cholinergic vasodilator nerves and their relation to the adrenergic vasoconstrictor nerves Acetylcholinesterase (AcChE) staining and the formaldehyde fluorescence method for the cellular localization of noradrenaline (NA) were used to visualize the two different types of vasomotor nerve terminal

[1] A preliminary report on this work has been published elsewhere (Bolme and Fuxe 1967)

Since the publication of our preliminary report on this subject (Bolme and Fuxe 1967), Schenk and El Badawi (1968) have suggested the existence of cholinergic nerve fibres in both large and small arteries from several tissues including skeletal muscle, heart, oesophagus and duodenum of cat and dog. These authors based their findings on the appearance of AcChE in nerve terminal-like structures.

Methods

Tissue samples from skeletal muscles were taken from 10 dogs and 8 cats, 2 human subjects and 3 monkeys (*Macaca irus*). In 3 cats and 3 dogs material from duodenum and oesophagus was also investigated (Table I). The samples were taken out *in vivo* and were immediately frozen in liquid propane cooled by liquid nitrogen. In order to visualize AcChE-containing structures, the tissue samples were cryostat sectioned and prepared according to Koelle's thiocholine method as modified by Holmstedt (1957). As an inhibitor of the unspecific cholinesterase, bismonoisopropylamido-phosphoryl fluoride (Mipafox) was used, whereas (p-N-allyl-N metylaminophenyl) pentan-3-one (Burroughs Wellcome (BW) 284c51) was used as an inhibitor of the specific AcChE. The slides were preincubated for 1/2 h in either Mipafox (4×10^{-6} M) or BW 284c51 (5×10^{-6} M) before incubation in the substrate solution at +37° C. After incubation, the sections were fixed with 10 % formalin at room temperature for 20—30 min. In some of the experiments cryostat sections were also treated according to the method described by El Badawi and Schenk (1967). In this modification of the technique described by Karnovsky and Roots (1964) tetraisopropylpyrophosphoramide (iso-OMPA) in a concentration of 8×10^{-4} M is used in the incubation medium to inhibit the unspecific cholinesterase. The incubation time used was 1 h at +37° C, before which the sections had been fixed for 15 min in 4 % formalin at +4° C. Adjacent tissue samples were freeze dried and treated with formaldehyde gas to visualize noradrenaline (NA), the adrenergic transmitter, using the histochemical fluorescence method for catecholamines (Falck *et al.* 1962, Hillarp, Fuxe and Dahlström 1966, Corrodi and Jonsson 1967).

In 3 dogs the lumbar sympathetic chain was extirpated on one side and 2—4 months later muscle samples were taken from both hind limbs for the cellular demonstration of NA and AcChE. In these experiments the microscopical evaluation was performed on coded preparations to avoid subjective errors.

Results

Dog. In samples from the gastrocnemius, the gracilis and the biceps muscles cholinesterase staining revealed precipitates of thiocholine in nerve terminal like structures mainly surrounding small intramuscular arteries 30—100 μ in diameter. The staining intensity was markedly reduced by BW 284c51 but not by treatment with Mipafox, indicating that the precipitates observed were derived from specific AcChE activity. In longitudinally sectioned vessels these AcChE-rich structures ("cholinergic terminals"), running parallel to the vessel wall, could be observed to exhibit enlargements, about 0.5—1 μ in size along the fibre. Similar results were obtained with the technique of Holmstedt (1957) and that of El Badawi and Schenk (1967).

The nerve terminals were located in the adventitia, often close to the media and were seldom observed to enter the superficial part of the muscular media (Fig. 1—3). A sparse plexus was present, built up of strands with varying numbers of AcChE-containing nerve terminals.

No AcChE activity was observed in relation to veins. Few if any, AcChE-rich terminals were found in association with large arteries (>100 μ) in limb skeletal muscle (Fig. 7). However, in the masseter muscle AcChE-rich terminals were observed around large arteries (Fig. 6). In contrast, around the small arteries (30—100 μ) in the masseter muscle, no AcChE-rich nerve terminals were found.

TABLE I Samples taken from various species and tissues for histochemical AcChE staining
H Holmstedt (1957) EB—S El Badawi and Schenk (1967) n Number of animals

Species	Tissue	Histochemical method (n)
Dog	gastrocnemius	H (10) EB—S (4)
	gracilis	H (6)
	biceps	H (2)
	masseter	H (3) EB—S (3)
	duodenum + oesophagus	EB—S (3)
Cat	gastrocnemius	H (8) EB—S (4)
	soleus	H (6) EB—S (4)
	duodenum + oesophagus	EB—S (3)
Monkey	gastrocnemius	H (3)
	gracilis	H (3)
	biceps	H (1)
Man	adductor magnus	H (1)
	tibialis ant	H (1) EB—S (1)



Fig 1



Fig 2

Fig 1 M gracilis of dog Stained for AcChE according to Holmstedt Incubation time 1 hr Preincubation with Mipafox AcChE activity → is found surrounding the media of an intramuscular artery which is longitudinally sectioned 300 ×

Fig 2 M gastrocnemius of dog Stained for AcChE according to Holmstedt Incubation time 1 hr A "cholinergic" nerve terminal → is observed in the adventitia of an intramuscular artery, which has been tangentially sectioned 480 ×





Fig 3 M gastrocnemius of dog Stained for AcChE activity according to Holmstedt Incubation time 2 hrs Transversely cut "cholinergic" fibres (→) are observed surrounding the media of a small artery 480 ×

Fig 4 M gastrocnemius of dog Fluorescence microphotograph Transversely cut adrenergic fibres are observed surrounding an intramuscular artery 480 ×

The noradrenaline (NA) containing nerve terminals (adrenergic nerve terminals) formed a ground plexus surrounding the outer layer of the media of the intramuscular arteries (Fig 4 and 5) The adrenergic ground plexus was observed around both large and small arteries Single strands of adrenergic terminals were occasionally found along small veins Furthermore the adrenergic nerve terminals were observed much more frequently than the ' cholinergic ' ones, even around the small arteries 30—100 μ in diameter

After unilateral lumbar sympathectomy no cholinergic or adrenergic nerve terminals were found around the muscle vessels on the operated side However on



Fig 5 M gracilis of dog Fluorescence microphotograph A longitudinally sectioned artery (A) and vein (V) are observed A strongly fluorescent adrenergic plexus is observed surrounding the intramuscular artery but not the vein 200 ×



Fig. 6. M. masseter of dog. Stained for AcChE activity according to El Badawi and Schenk. Incubation time 1 hr. A rich "cholinergic" plexus is observed surrounding the media of an intramuscular artery ($>100\ \mu$). Strands with single (→) or several (|→) fibres are found. 300 X

Fig. 7. M. gastrocnemius of dog. Stained for AcChE activity according to El Badawi and Schenk. Incubation time 1 hr. No AcChE activity is observed close to the artery ($>100\ \mu$). 300 X



Fig. 8. M. gastrocnemius of cat. Stained for AcChE according to El Badawi and Schenk. Incubation time 1 hr. A small artery (A) and two small veins (V) are seen. A transversely sectioned "cholinergic" fibre is observed (→) in the adventitia of the artery. 300 X

the intact side the appearance of both "cholinergic" and adrenergic nerve terminals around vessels was normal.

Samples were also taken from the oesophagus and duodenum. With the technique of El Badawi and Schenk (1967) AcChE rich nerve terminals were observed in these tissues surrounding both small and large arteries.

Cat. Muscle samples were taken from the gastrocnemius and the soleus muscles of the cat to study the vasomotor nerve arrangement in both white and red muscle. In both muscles the localization and density of adrenergic and "cholinergic" (Fig. 8) nerve terminals appeared similar to that seen in the dog muscle. As in the dog the

adrenergic nerve terminals were observed more frequently than the AcChE-rich ones

Samples were al o taken from the oesophagus and duodenum Microscopy revealed AcChE rich nerve terminals with the method of El Badawi and Schenk (1967) sur rounding both large and small arteries

Man and monkey Cryostat sections from muscle samples were examined but in no case did we find any AcChE rich nerve terminals around the vessels However adrenergic nerve terminals were seen in adjacent muscle samples They were localized around the vessels of the muscle in the same way as described for cat and dog

Discussion

The following histochemical observations indicate the existence of sympathetic cholinergic nerve terminals around small arteries in skeletal muscle of cat and dog

1 High AcChE activity was found close to the vessel wall
2 The AcChE activity was localized in nerve terminal like structures with AcChE rich enlargements along the fibre which have been observed previously in iden tified autonomic cholinergic and adrenergic nerve terminals (Hillarp 1946 Falck 1962 Norberg and Hamberger 1964 Ehinger 1966)
3 The AcChE activity disappeared after chronic sympathectomy

The histochemical identification of cholinergic nerve terminals is indirect and based on the demonstration of the acetylcholine hydrolyzing enzyme acetylcholin esterase (AcChE) in nervous structures At present it is not possible to identify *histochemically the cholinergic transmitter acetylcholine itself Until such direct* methods have been developed our knowledge about cholinergic innervation must be based on the demonstration of AcChE and/or physiological studies The rationale for using histochemical criteria has been thoroughly discussed (Fredriksson and Sjoqvist 1962 Sjoqvist 1962 Jacobowitz and Koelle 1965)

In the present experiments it cannot be excluded that the AcChE activity was localized to some of the adrenergic nerve terminals since simultaneous demonstra tion of AcChE and NA was not performed in the same section (*cf* Giacobini Palm borg and Sjoqvist 1967 Buckley *et al* 1967) However this is unlikely since there are findings indicating that adrenergic nerve terminals lack detectable amounts of AcChE activity at least in the rat (Ehinger and Falck 1966) and since the fluo rescent probably adrenergic ganglion cells in lumbar ganglia of cat contain very little if any AcChE activity (Hamberger Norberg and Sjoqvist 1965) Further more we observed fluorescent adrenergic nerve terminals much more frequently than AcChE rich ones and it is hard to explain why AcChE might have been visualized in only a small proportion of the adrenergic neurons Therefore circum stantial evidencies indicate that the AcChE rich nerve terminals observed really were cholinergic and that those found in connection to small arteries in skeletal muscle represented the sympathetic cholinergic vasodilator nerves

In both cat and dog similar arrangements of the cholinergic and the adrenergic nerve terminals were observed, mainly restricted to the adventitia surrounding the muscular media (*cf* Falck 1962, Fuxe and Sedvall 1965) However, cholinergic nerve terminals, as revealed by the intense staining of AcChE, were found much less frequently than adrenergic terminals Therefore, the observation made by Folkow Öberg and Rubinstein (1964) and by Bolme, Ngai and Rosell (1967) that a high prevailing vasoconstrictor tone reduces the response on vasodilator nerve stimulation, may be explained by the apparent quantitative difference in adrenergic and cholinergic innervation

A difference was observed in the distribution of cholinergic nerve terminals between limb skeletal muscle and the masseter muscle of the dog No AcChE rich terminals were seen surrounding small arteries in the masseter muscle This finding is in agreement with the observation by Bolme and Edwall (1969) that vasodilatation was not obtained in the masseter muscle following stimulation of hypothalamic vasodilator nervous pathways On the other hand, in the masseter muscle AcChE rich nerve terminals were observed around large arteries (>100 μ) The function of these nerves cannot be interpreted at present The same difficulty arises when explaining the significance of the AcChE rich nerve terminals seen around arteries of different dimensions in the duodenum and oesophagus, also observed by Schenk and El Badawi (1968) No physiological data are available which could indicate the function or origin of these nerves

No difference in the distribution of cholinergic nerve terminals was observed between white and red muscle of the cat (gastrocnemius and soleus muscle respectively) This finding is in accordance with the results of Barlow and Walder (1965) They obtained vasodilatation of comparable magnitudes from white and red muscle of the cat following sympathetic vasodilator nerve activation

It was not possible to demonstrate any AcChE rich nerve terminals around skeletal muscle vessels of man and monkey This finding has to be interpreted with great caution since the specificity and the sensitivity of the AcChE methods used in the present study are not tested in these species However, there are supporting physiological findings (Bolme Novotny Uvnas and Wright 1969) indicating that in the monkey skeletal muscle vessels of the hind limb are not innervated by sympathetic cholinergic vasodilator nerves

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Species Distribution of Sympathetic Cholinergic Vasodilator Nerves in Skeletal Muscle

By

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Abstract

BOLME P NOVOTNY, J UVNÄS B and WRIGHT, P G, *Species distribution of sympathetic cholinergic vasodilator nerves in skeletal muscle* Acta physiol scand. 1970 78 60—64

The lumbar sympathetic chain was electrically stimulated in different species before and after blocking the adrenergic vasoconstrictor nervous response Blood flow in the hind limb skeletal muscles was measured In all species studied fox sheep goat monkey (five different strains) polecat rat badger opossum rat and hare stimulation of the lumbar chain before adrenergic blockade resulted in a vasoconstriction After blocking the vasoconstrictor nervous response stimulation elicited a blood flow increase in fox sheep and goat After atropine the response to stimulation was blocked indicating that sympathetic cholinergic nerves had been activated In the other species studied no vasodilator response was observed upon sympathetic chain stimulation The results suggest that the role attributed to the vasodilator nerves anticipatory to muscle exercise are played by other mechanisms in species lacking sympathetic cholinergic vasodilator nerves

The distribution of sympathetic cholinergic vasodilator nerves to skeletal muscle vessels of different species has not been systematically investigated The existence of these nerves in dog and cat was reported by Bulbring and Burn (1935 1936) and later confirmed by Folkow and Uvnäs (1948 1949) and Folkow Haeger and Uvnäs (1948

The existence of sympathetic cholinergic vasodilator fibres have been reported by Fray and Leaders (1967 in the rat and by Feigl and Folkow (1963) in the duck On the other hand Bulbring and Burn 1936) could not demonstrate any sympathetic cholinergic vasodilatation in skeletal muscle of hare, rabbit or rhesus monkey

In view of the relatively sparse data available on sympathetic cholinergic vasodilator innervation in different species we decided to study this problem more

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systematically Furthermore, the physiological significance of these nerves is not clear and by the present investigation it was hoped to obtain further information about the role played by the sympathetic cholinergic vasodilator nerves

Methods

The experiments were performed on the following species fox (*Vulpes vulpes*), hare (*Lepus timidus*), badger (*Meles meles*), goat (*Capra domestica*), sheep (*Ovis domesticus*), polecat (*Mustela putorius*) rat (*Sprague Dawley*) and opossum rat (*Didelphys virginiana*) In addition different varieties of monkey were tested baboon (*Papio anubis tessellatus*), de Brazza [illegible] (*Macaca irus*) patas (*Erythrocebus patas*) and vervet [illegible] with pentobarbital (10—60 mg/kg i p or i v) or [illegible] and/or chloralose (30—70 mg/kg i v)

The arterial pressure was recorded in the carotid artery and usually measured by a Statham pressure transducer (P 23 AC) In some experiments a mercury manometer was used instead The blood flow was measured with a venous or arterial drop recorder (Lindgren 1958) after heparinization (1000—5000 I U /kg i v) In rat and opossum rat the abdominal aorta was cannulated and the blood flow to the entire hind quarters of the animal was measured In all other species studied the blood flow to a muscle preparation was determined Depending on the size of the animal either the isolated gastrocnemius muscle or the skinned hind limb was used All recordings were made on a Grass model 5 polygraph or, in some experiments on [illegible] al or by the retroperitoneal [illegible] stimulated at the L 2—L 5 [illegible] stimulator and consisted of [illegible] imp/sec and intensities of 2—10 V In some of the monkeys electrical stimulation with unipolar electrodes was performed in the hypothalamus using stereotactic technique The stimulation parameters were intensity 5—30 V, frequency 50—100 imp/sec and duration 1—10 msec

The sympathetic vasoconstrictor nervous response was blocked with different drugs The [illegible]

Results

Stimulation of the lumbar sympathetic chain produced vasoconstriction in all animals except those pretreated with reserpine No obvious differences between the species were observed neither in the magnitude of the constrictor response nor in the stimulus parameters needed to produce vasoconstriction When the vasoconstrictor response had been tested, the adrenergic blocking agent was administered A representative experiment is shown in Fig 1 The sympathetic chain was stimulated in the goat and a vasoconstriction was elicited (left panel) After the administration of dihydroergotamine—in this animal a very low dose (0 1 mg/kg i v)—the response to stimulation was reversed to a vasodilatation (middle panel) The dilatation was blocked by atropine (0 1 mg/kg i v) The stimulation after atropine resulted in a slight vasoconstriction indicating that the adrenergic vasoconstrictor nervous response was not completely blocked

The result of the study is summarized in Table I With the technique used th

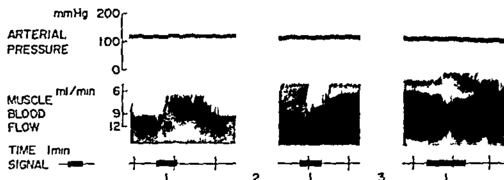


Fig 1 Goat 18 kg Chloralose anesthesia M gastrocnemius 140 g Effect of stimulation of lumbar sympathetic chain
1 Stimulation with 8 V 5 msec 10 imp/sec
2 Dihydroergotamine 0 1 mg/kg i.v
3 Atropine 0 1 mg/kg i v

presence of sympathetic cholinergic vasodilator nerves in skeletal muscle of fox sheep and goat was indicated In the other species studied, and among these many types of monkey, we were unable to find evidence for such nerves Furthermore in five monkeys (vervets, not included in the table) electrical stimulation was performed in the hypothalamus by systematically moving the electrode in one mm steps horisontally and vertically Muscular vasodilatation was never obtained by this procedure After the experiments the brains were examined macroscopically to test that stimulation had been performed in the hypothalamus

Discussion

In the present study experimental results have been presented indicating that species differences exist in the occurrence of sympathetic cholinergic vasodilator nerves in skeletal muscle Our findings agree with those of Bülbring and Burn (1936) that monkey and hare lack sympathetic cholinergic vasodilator nerves Similarly, Bolme

TABLE I Distribution of sympathetic cholinergic vasodilator nerves to skeletal muscles of various species *n* number of animals tested

n	Vasodilatation obtained	n	No vasodilatation obtained
4	Sheep		Monkey
5	Goat	8	Vervet
4	Fox	3	Baboon
		2	de Brazza
		2	Patas
		3	Cynomolgus
		4	Polecat
		8	Badger
		5	Opossum rat
		3	Rat
		6	Hare

and Fuxe (1969), using histochemical methods obtained results indicating species differences in the innervation of arteries in skeletal muscles, cholinesterase rich nerve terminals were found in the wall of small arteries of cat and dog but not of man and monkey (*Macaca irus*)

Folkow and Rubinstein (1966) reported that topical stimulation into the hypothalamic defence area in the rat did not produce any cholinergically mediated vasodilatation in the hind limb as seen in cat and dog indicating that the rat lacks sympathetic cholinergic innervation of muscle vessels This finding is in agreement with our results On the other hand Fray and Leaders (1967) obtained vasodilatation in the hind quarters of the rat upon lumbar sympathetic chain stimulation after blocking the adrenergic vasoconstrictor nervous response Those authors interpreted their results as indicating that sympathetic cholinergic vasodilator fibres exist in the rat However, the vasodilatation observed was small (10 per cent decrease in perfusion pressure) and the response after atropine was not tested It cannot be ruled out that in the experiments of Fray and Leaders (1967) the vasodilatation obtained was caused in other ways than by activating cholinergic vasodilators nerves *e g* by stimulating adrenergic β receptors (*cf* Viveros Garlick and Renkin 1968)

The differences in distribution of cholinergic vasodilator nerves between different species do not seem to contribute to the understanding of the physiological importance of these nerves Among species in which sympathetic vasodilator nerves were found there are both predatory and ruminant animals *e g* fox and goat respectively with no obvious common qualities which could explain why these very species need special vasodilator nerves to their skeletal muscle vessels

It has been suggested that the sympathetic cholinergic vasodilator nerves are activated in emotional situations and anticipatory to muscular effort (Eliasson *et al* 1951 Abrahams Hilton and Zbrozyna 1960 1964 Bolme and Novotny 1969) There is little reason to believe that the animals in which no sympathetic vasodilator nerves were found lack the ability to increase the blood supply to their skeletal muscles in emotional situations or prior to exercise Smith and Stebbins (1965) reported for instance that in monkeys the blood flow in the abdominal aorta increased suggesting a vasodilatation in skeletal muscles as conditional response to an electrical cutaneous shock Since no sympathetic vasodilator nerves to skeletal muscles of monkey have been found the blood flow increase observed by Smith and Stebbins (1965) was probably caused in other ways than by vasodilator nerve activation

The existence of sympathetic cholinergic vasodilator nerves in skeletal muscle of man have been claimed by several authors (Blair *et al* 1959 Barcroft *et al* 1960 Greenfield 1965) However the evidence for vasodilator nerves in man is with necessity circumstantial Our findings that monkeys among many animals lack vasodilator nerves make us believe that the indications for the presence of sympathetic vasodilator nerves in man must be critically judged Until more direct evidence is presented the question whether or not sympathetic cholinergic vasodilator fibres exist in skeletal muscle of man cannot be answered

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Extraction of Dopa from the Integument of Pigmented Animals

By

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Abstract

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The occurrence of high amounts of dopa has been demonstrated chemically in the piliary system of pigmented animals (guinea pig rabbit, and dog) Histochemically, a formaldehyde induced fluorescence was recorded only in melanocytes of hair bulbs The spectral characteristics of these cells did not agree with those of the authentic dopa fluorophore The discrepancy between the chemical and histochemical findings is discussed Little or no dopa was found in non pigmented skin of albino and multi-coloured guinea pigs This indicates that the integumentary dopa is related to the pigment formation

It has long been well established that 3 4-dihydroxyphenylalanine (dopa) is an intermediary in the biosynthesis of catecholamines and melanin Significant amounts of dopa, however, have not until recently been detected in mammalian tissues, and it was thought that it is not accumulated but rapidly metabolized By means of the histofluorescence technique of Falck and Hillarp a formaldehyde induced fluorescence was demonstrated in human melanocytes and nevus cells (Falck *et al* 1965 1966a) On the basis of present knowledge regarding the synthesis of melanin on the one hand, and the molecular structure required for a substance to form a fluorophore with formaldehyde, on the other, (Corrodi and Jonsson 1967) this cellular fluorescence was thought to reflect the presence of dopa (Falck *et al* 1965) Recently, the presence of dopa in experimental melanoma (Takahashi and Fitzpatrick 1966) and in human malignant melanoma cells (Falck *et al* 1966 a and b) has been demonstrated and dopa has been detected chemically in pooled samples of a great number of pigmented nevi (Cegrell Falck Jacobsson and Rosengren unpublished results) Moreover Bernheimer (1963) found dopa in cattle eyes Ehinger and Rosengren (1967) could establish the presence of dopa in the ciliary epithelium and retinal pigment cells of albinotic embryos of some mammals and Cegrell *et al* (1967) found dopa in skin of young pigmented mice To obtain further evidence of a storage of

TABLE I. Dopa content in hair and shaved skin in different species. The figures express M±SEM or values of single determinations

Species	Extraction agent	Number of determinations	Dopa μg/g wet weight	
			Hair	Shaved skin
Pigmented guinea-pigs	boiling water	7	2.1±0.41	0.29±0.10
	perchloric acid	7	0.90±0.16	0.10±0.007
Albino guinea pigs	boiling water	2	0.09 and 0.14	0.03 and 0.03
Multi coloured guinea pigs				
white parts	boiling water	2	0.14 and 0.22	0.00 and 0.02
coloured parts	boiling water	2	3.4 and 5.3	0.27
Pigmented guinea pigs	ethanol	2	0.55 and 0.97	—
Pigmented young rabbits	boiling water	4	8.5, 12, 23, 24	1.9 and 3.8
	perchloric acid	2	2.4 and 5.7	0.76 and 0.8
Pigmented adult rabbits	boiling water	1	1.3	0.05
	perchloric acid	1	0.46	0.02
Pigmented dogs	perchloric acid	3	4.6, 4.7, 5.6	0.63 and 1.0

dopa in the mammalian skin the present study was undertaken. It disclosed that considerable amounts of dopa can be found in the integument of some species but that high quantities were present in a quite unexpected location, i.e. the hairs.

Material and methods

The material is composed of 13 young guinea pigs, 4 young and 1 adult pigmented rabbits (grey and dark) and 3 young black dogs (4 weeks of age).

Two guinea pigs had both multi-coloured and non pigmented skin areas (below called multi-coloured animals), 9 were fully pigmented and 2 were albinos. They were killed by decapitation and the hair of most of the body integument was removed with electric clippers. The hair from each of 7 pigmented animals was divided into two approximately equal samples as was the clipped skin (in the following called "shaved skin"). One sample of each tissue was extracted with boiling water for 5 min and the other was extracted with perchloric acid. Four samples consisting of coloured and non coloured hair and shaved skin, respectively, were collected from each of the multi-coloured animals. These samples were extracted with boiling water for five min. In two albino guinea pigs the body integument was divided into hair and shaved skin and extracted with boiling water. Finally, extraction with ethanol was performed on the hair from two pigmented animals.

The rabbits were killed by an intravenous injection of air, the dogs by bleeding in pentobarbital anesthesia. Samples of hair and shaved skin were obtained from suitable skin areas. The extraction was performed with either boiling water (5 min) or perchloric acid (see Table I).

The catechol derivatives were fluorimetrically determined according to Anton and Sayre (1964) and in some cases according to Ehrlén (1948). For further identification of the catechol derivatives the Al_2O_3 eluates (guinea pig) were chromatographed in three different systems: butanol-benzene-methanol-H_2O (4:4:4:1), butanol-N HCl and phenol-0.1 N HCl (9:1) (cf. Bertler et al. 1958). The catechol derivatives were visualized by spraying the papers with potassium ferrocyanide or by exposing the papers to dry formaldehyde gas (generated from paraformaldehyde) at 80° C for 1 hr.

Two samples of pigmented hair from two young rabbits were boiled in water (5 min) and the extracts obtained were incubated at 37° C for 30 min together with pyridoxal-5-phosphate

and an extract of rabbit kidney cortex (see Bertler and Rosengren 1959) The amount of dopamine formed under these conditions was determined according to Bertler *et al* (1958)

At least two small tissue specimens from the shaved skin of each animal were taken for histochemical analyses according to the fluorescence method of Falck and Hillarp (for technical details see Falck and Owman 1965) The microspectrofluorimetric analyses were carried out with a modified Leitz' microspectrograph as reported by Björklund *et al* (1968)

Results and comments

The results of the chemical determinations according to the method of Anton and Sayre (1964) are summarized in Table I Both the hair and the shaved skin from the pigmented animals contain significant amounts of a substance that irrespective of the extraction procedure behaved like dopa in these fluorimetric analyses It also appeared that those samples of guinea pig hair treated by the trihydroxyindole method of Ehrlén (1948) yielded a fluorescence typical for dopa and contained quantities of dopa that agreed with those found with the periodate method Moreover, on the paper chromatograms a spot could be visualized with either $K_3Fe(CN)_6$ or exposure to formaldehyde gas In the three systems used this spot displayed the same Rf values, colour, and fluorescence as the spot of authentic dopa which was used in all the experiments as reference substance Finally in the two decarboxylation experiments with rabbit hair a formation of dopamine occurred The dopamine found after decarboxylation was of the same order of magnitude as that obtained in model experiments in which dopa was decarboxylated under as identical conditions as possible The recovery in these studies is about 10—15 % and the amount of dopamine formed was 1 6 and 0 85 μg/g which corresponded well to the amount of dopa in hair, 12 and 8 5 μg/g respectively Thus it seems well established that dopa is present in the pilary system of pigmented animals

The mild extraction procedures applied in this study can hardly release dopa from dopa-containing proteins and with respect to melanin, it seems quite excluded that a hydrolysis can occur It is thus reasonable to suppose that the demonstrated dopa exists in free form Fitzpatrick (1965) found dopa in melanosomes but only after hydrolysis of trichloracetic precipitates of the organelles with strong hydrochloric acid He concluded that this dopa was present within the protein molecule

It is evident from Table I that extraction with boiling water for 5 min gives a much higher yield of dopa than that obtained by perchloric acid which is otherwise a more favourable agent for the extraction of dopa, thus, for example more dopa can be extracted from an islet cell tumour of golden hamster with this acid than with boiling water (Cegrell Falck and Rosengren unpublished observations The difference between perchloric acid and water at 100° C as extracting agents can possibly be explained by the fact that in the present case dopa had to be extracted from a keratinized tissue Ethanol proved also to be an unfavourable extraction agent which could be confirmed in recovery experiments on other tissues as well (dopa added to brain tissue)

The concentrations of dopa found in the pigmented hair are high especially high values were recorded in young rabbits In the shaved pigmented skin the values were

considerably lower However, this obviously does not necessarily imply that the concentration of dopa is lower in the pilary system below the epidermal surface In the albino guinea pigs and in the non pigmented integumental parts of the multicoloured guinea pigs, comparatively slight amounts of dopa were found This strongly indicates that the integumentary dopa has a relation to pigment formation

In the fluorescence microscope, the hair showed an intense autofluorescence and the possible presence of any specific, *i e* formaldehyde induced fluorescence could not be evaluated In agreement with previous results from studies on guinea pig skin (Olivecrona and Rorsman 1966) the hair matrix in the pigmented skin of the guinea pigs and rabbits was found to contain melanocytes which displayed a specific granular green yellow fluorescence The number of fluorescent cells in the hair bulbs showed great intra and inter individual variations and in some animals including all the dogs, no fluorescence, but only cells heavily loaded with pigment could be seen In the pilary system of albino animals, neither pigment granules nor fluorescent granules were observed

In the microspectrograph the specifically fluorescent melanocytes showed excitation spectra with maxima at about 430 mμ whereas the maxima of the emission curves varied between 480 and 520 mμ Measured under the same conditions the fluorophore of authentic dopa showed excitation/emission maxima at 410/480 mμ It seems unlikely that the difference in the emission spectra is caused by absorption in pigment granules since measured cells appeared colourless in transmitted light Whether the formaldehyde-induced fluorescence reflects the presence of one or more fluorophore forming substances with or without concomitant occurrence of dopa cannot at present be evaluated The variations in emission maxima suggest the presence of a mixture of fluorophores in different proportions Human malignant melanoma cells are known to contain at least two fluorogenic substances of which only dopa has so far been identified (Falck *et al* 1966) and display spectral characteristics (Ehinger *et al* 1968 Cegrell Falck and Rosengren unpublished observations) similar to those obtained from the melanocytes in this study

The biological significance of dopa in the hair shaft is obscure It is tempting however to suggest that it is incorporated in connexion with uptake of pigment granules into the hair

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A Comparison of Blood Viscosity Measured In Vitro and in a Vascular Bed

By

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Abstract

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Blood viscosity *in vivo* ('apparent viscosity') and its variations with flow rate was analyzed in the maximally dilated calf muscle vascular bed of the cat by comparing pressure flow relationships for blood and a Newtonian fluid (dextran Tyrode) over a flow range between 60 and 0.2 ml/min × 100 g tissue. Viscosity *in vitro* for the same perfusates was measured in a cone plate viscometer. — Apparent viscosity was much lower (approximately 50 %) than *in vitro* values at high shear rates, with less variation between animals. It increased with de [illegible] which occurred at flows around 0.5 ml/min [illegible] in constricted vessels with higher flow ve[illegible] [illegible] f viscosity changes with flow in the intact [illegible] p rise of viscosity *in vitro* at quite low shear rates had no counterpart *in vivo*, in fact, viscosity then tended to fall again. The discrepancies between blood viscosity *in vivo* and *in vitro* seem to be related to vascular dimensions favouring "bolus flow" and hence low regional viscosity in the most narrow vessels, which may become more pronounced at further luminal reduction, active or passive.

Addition of high molecular weight dextran (HMD) raised apparent viscosity to seemingly high levels at low flows. However, to a considerable extent this appears to be due to cell aggregation and plugging of microvessels rather than to a genuine increase of blood viscosity.

Studies *in vitro* show that blood viscosity decreases considerably with tube radius once this is below 60—100 μ (Fåhraeus and Lindqvist 1931), to approach plasma values when capillary dimensions are reached (Bayliss 1952, Prothero and Burton 1962). Further, due to the anomalous non-Newtonian flow properties of blood viscosity tends to increase with reduced flow velocity. Therefore under normal physiological conditions viscosity may be expected to decrease continuously towards the capillary level, and then to increase again in the veins to values even higher than

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in the arteries due to the low flow velocity in the wide bore venous section Consequently the *average* blood viscosity *in vivo* (*"apparent viscosity"*) must be a complex function of both regional vascular design and flow rate (*cf* Haynes 1961, Whitmore 1968)

The flow resistance offered by each consecutive section of a vascular bed is the *product* of the hindrance inherent in its design *and* the regional blood viscosity total flow resistance is the *sum* of all these products Since the major portion of the total flow resistance resides in the arteriolar section, the apparent viscosity must be largely determined by the regional viscosity in this section, which is presumably low The supposedly high regional blood viscosity in the venous compartment will have little influence on the apparent viscosity, since flow resistance in these vessels is low However, an increase in venous blood viscosity might considerably increase the post-capillary flow resistance without altering the *total* flow resistance much and in this way significantly affect transcapillary fluid transfer and hence plasma volume

In agreement with the above considerations it has been shown that blood viscosity *in vivo* (*i e* apparent viscosity) is considerably lower than that measured *in vitro* (Whittaker and Winton 1933 Levy and Share 1953, Olsson 1964) The vascular bed is of course, the most relevant of all viscometers but can be used as such only if all other variables affecting flow are rigorously controlled In particular this applies to the active and passive changes of vascular dimensions that follow alterations in perfusion pressure and flow

A vascular bed which displays a linear pressure flow relationship would apparently offer ideal conditions for the measurement of viscosity Such a relationship, however, suggests in fact that active vascular smooth muscle adjustments take place to counteract the physical distention of the resistance vessels at higher pressures and flows (Folkow and Löfving 1956) Comparisons of pressure flow characteristics of blood and Newtonian fluids for viscosity measurement then become hazardous, since smooth muscle tone might not be identical with the two perfusates Therefore if the vascular bed is to be used as a viscometer, *all* smooth muscle activity should be abolished and the passive elastic behaviour of the vascular bed fully displayed

It is not impossible that the presence of some smooth muscle activity may have affected the results of some of the earlier studies on blood viscosity *in vivo* since a linear pressure flow relationship was often obtained Furthermore to our knowledge measurements of blood viscosity in vascular beds have not been performed at very low blood flows, since perfusion pressures below 30 mm Hg do not seem to have been used This is, however, necessary in order to explore whether the marked increase of blood viscosity observed *in vitro* at very low shear rates is of relevance for the situation in a vascular bed at very low rates of flow

The present study was undertaken to examine blood viscosity *in vivo* with particular attention to low rates of flow and to compare the results with viscosity measurements *in vitro* The completely isolated vascular bed in the calf muscles of the cat was chosen as the biological viscometer Intermittent supramaximal muscular work was used tc

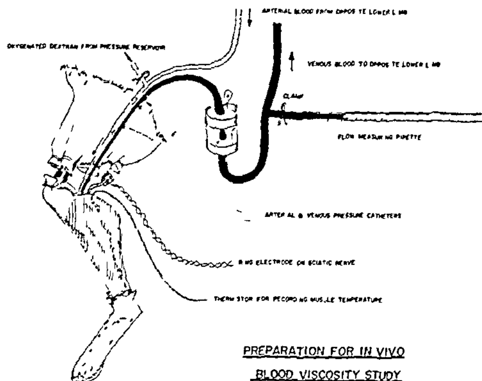


Fig. 1. Schematic illustration of the isolated calf muscle preparation used as the biological viscometer.

checked by the addition of large doses of vasodilator drugs. At well-defined distending pressures and consequently comparable vascular dimensions, the pressure-flow relationships for blood and a Newtonian fluid (oxygenated dextran Tyrode solution) were compared. Differences in flow values at identical perfusion pressures permit calculation of blood viscosity *in vivo* over a wide range of flows. A preliminary report of this study has been presented earlier (Djojosugito *et al.* 1968).

Methods

Cats ranging from 3—5 kg in weight were anesthetized with ether and maintained with intravenous chloralose (30—50 mg/kg b.w.).

Operative procedures

The preparation of the isolated calf muscle group and the plethysmographic technique for the determination of volume changes were carried out as described by Kjellmer (1964) with small modifications (Fig. 1). The paw of the hind limb was disarticulated at the ankle joint and the tendons and skin at that point fixed to bone with strong ties. A circumferential skin incision was made at the upper thigh and the skin dissected free from underlying fascia distally in one piece down to the ties at the ankle joint. The collar of the avascular skin "sock" so formed was used to seal the plethysmograph when experiments concerned with volume changes were carried out. The femoral artery and vein were dissected free and isolated by tying all branches as far distally as the popliteal fossa. The thigh muscles were divided by thermocautery immediately above their respective distal insertions about the knee joint, a hole was drilled in the lower femur and the marrow cavity packed with vaseline gauze. The sciatic nerve was divided. Following heparinization (5 mg/kg) small side branches of the popliteal artery and vein

close to the muscle group, were cannulated to record arterial inflow and venous outflow pressures. Wide bore polyethylene catheters were introduced more proximally into the femoral arteries and veins of both lower limbs in such a way that the 'test' limb was perfused and drained by the vessels of the opposite limb. The isolated muscle could also be perfused with [illegible] from a pressure bottle connected to a side branch of the [illegible] enous [illegible] ments were made using an almost horizontally held calibrated pipette connected to the venous catheter via a T-connection proximal to the drop chamber. By appropriate simultaneous adjustment of haemostats the venous outflow could be directed into the pipette, from which flow per unit time was obtained by "timing" the movement of the meniscus between marks.

A thermistor was placed beneath the skin "sock" on the muscle to monitor temperature of the preparation, which was maintained at approximately 37° C by warm saline soaked packs and the judicious use of heat lamps or the thermostatically controlled warm water in a plethysmograph. The muscles were made to exercise by stimulation of the cut peripheral end of the sciatic nerve using a ring electrode and Grass stimulator, delivering pulses strong enough to activate all motor units but well below the threshold of the posteanglionic vasoconstrictor fibres. The stimulation frequencies used were carefully tested to produce a maximal vasodilatation at each level of perfusion pressure. At the end of the experiment, the muscle group was dissected free and weighed.

Measurements of circulatory variables

Arterial and venous pressures were recorded in the popliteal artery and vein using Statham strain gauges and a Grass model 7 Polygraph. The strain gauges were calibrated throughout each experiment against mercury and water manometers and mean pressures during the experiment were determined by electronic damping. The zero reference plane for all pressures was an arbitrary plane passing through the manubrium sterni of the supine cat. The height of the pipette in the venous circuit was adjusted so that when blood was diverted for flow recordings the pre-diversion venous pressure in the popliteal vein was maintained.

Blood flow, expressed as ml/min × 100 g tissue, was measured when steady state flow was established, as evident from the drop recorder registration. The venous effluent was then diverted to the pipette and the movement of the fluid meniscus between marks on the pipette timed using a stop-watch. Only at high rates of flow the accuracy of the technique was limited by the observers ability to accurately define the instant when the fluid meniscus passed the mark. However, at a perfusion pressure of 70 mm Hg the greatest deviation from the mean of 5 consecutive flow determinations was less than 1 per cent of the mean flow value (38.7 ml/min × 100 g tissue).

For measurements of alterations in skeletal muscle volume and capillary filtration coefficient (CFC), the leg was placed in a perspex plethysmograph where it was fixed firmly by screw clamps gripping the ankle. The plethysmograph was sealed with the skin collar that previously covered the thigh, and filled with water maintained at 38° C. The volume changes in the plethysmograph were measured with an air filled piston recorder giving a deflexion of 50—60 mm/ml. The venous outflow circuit was interrupted between the optical drop recorder and the opposite femoral vein to permit alterations in the height of the opening of the draining catheter above the muscle. In this way the venous outflow pressure could be suddenly raised, usually by 10—20 cm H_2O, for determination of the capillary filtration coefficient (CFC).—The technique of CFC measurement and the method of calculation has been fully discussed in previous publications (Cobbold, Folkow, Kjellmer and Mellander 1963). For the calculation of CFC it was assumed that 80 per cent of the change in venous pressure (as measured from the venous pressure strain gauge) was transmitted to the capillaries. Recordings of volume changes were made on smoked paper on a slowly moving kymograph drum.

Viscosity measurements

Viscosity *in vitro* was determined in a Wells-Brookfield micro cone plate viscometer model LVT (Brookfield Engineering Laboratories Inc. Stoughton, Mass.) using 1 ml samples. Values were expressed as absolute viscosity measured to an accuracy of ±0.1 centipoises, the reproducibility of measurements varied with the shear rate used. For 4 determinations of viscosity taken from a single sample of blood the greatest deviation from the mean at shear rates of 23 sec^{-1}, 46 sec^{-1}, 115 sec^{-1} and 230 sec^{-1} was 8, 4, 3 and 0 per cent of the mean viscosity value respectively.

Values for blood viscosity *in vivo* were obtained by constructing and comparing pressure-flow curves for blood (autoperfusion from the opposite femoral artery) and for oxygenated dextran Tyrode solution, a Newtonian fluid. Pressure changes were effected by manipulating

a screw clamp on the arterial polyethylene catheter proximal to the site for the pressure recording Flow measurements were always made immediately following 1/2—1 min period of "supramaximal' exercise, at a time when flow (as indicated by the optical drop recorder) and arterial and venous pressures were stable Failure to produce further vasodilatation with close arterial injections of huge doses of isoprenaline and acetylcholine (25—50 µg) indicated that a maximal vasodilatation was produced by the exercise Preliminary studies on tissue volume changes during the experimental procedure indicated that during the initial few minutes of exercise at the commencement of the experiment the limb increased in volume but rapidly stabilized *All pressure flow curves were obtained at this stabilized volume* '*Hysteresis*' was often fairly marked both in the blood and dextran Tyrode curves, but was avoided as a complicating factor in the analysis of data by commencing flow measurements for each curve at an arterial pressure of 40 mm Hg then proceeding with a standard sequence of flow deter

(HMD) were determined the HMD (2 5 ml/kg of 15 per cent solution) was given intravenously and allowed to mix in the animal for 30 min The pressure flow curve was then obtained by autoperfusion as for normal blood Efforts were made to keep the calculated mean *transmural distending pressure (arterial plus venous pressure divided by 2) for the vascular bed similar for each perfusate at any given perfusion pressure over the whole range of pressures used* This is important if identical vascular dimensions at a given perfusion pressure is desired since the vascular bed showed a marked distensibility at least with perfusion pressures below 80 mm Hg

Results

Complete pressure flow curves (with the vascular bed in a maximally dilated state following intermittent exercise) were established for normal blood and dextran Tyrode solution in 6 entirely successful skeletal muscle preparations In 4 of these experiments additional curves were obtained for blood after administration of high molecular weight dextran (HMD) to the animals Relevant data from all experiments are presented in Table I and mean pressure flow curves from pooled data are shown in Fig 2

For each perfusate there were only small discrepancies between the pressure flow curves from the different individual experiments indicating a very uniform basic structural design of the vascular bed in the calf muscles All curves including those for dextran Tyrode solution were convex towards the pressure axis implying a considerable distensibility of the resistance vessels particularly in the lower range of transmural pressures This distensibility was also demonstrated by the fact that elevation of arterial inflow and venous outflow pressures by an equal amount resulted in an increased blood flow despite an unchanged pressure head These findings agree with those of Folkow and Lofving (1956)

None of the pressure flow curves showed any intercept on the pressure axis Even when blood mixed with high molecular weight dextran (HMD) was perfused at low pressures flow continued as long as there was a measurable pressure head This was not due to incorrect pressure recordings since the calibration of the recording devices were checked frequently However the flow measured in the venous catheter at very low perfusion pressures might at least partly represent tissue fluid absorbed in the capillaries as a consequence of the reduced capillary pressure To test

TABLE I Viscosity data from experiments on the maximally dilated calf muscle vascular bed of the cat, perfused with dextran Tyrode solution, normal blood and blood mixed with high molecular weight dextran (HMD) The values for viscosity *in vivo* were deduced from flow values at a perfusion pressure of 80 mm Hg and indentical transmural pressures, the *in vitro* values were determined in the cone plate viscometer at high shear rate (230 sec^{-1}) — Values for hematocrit (Hct) and erythrocyte sedimentation rate (ESR) are also included

			Viscosity, centipoise						
Exp no	Htc %	ESR mm/hr	Dextran Tyrode in vitro	Plasma in vitro	Blood in vitro	Blood in vivo	Plasma +HMD in vitro	Blood +HMD in vitro	Blood +HMD in vivo
1	37	90	3 1	—	5 7	2 5	—	—	—
2	47	5	2 8	1 2	5 6	2 5	—	—	—
3	41	8	3 1	—	5 3	2 3	2 9	6 1	4 4
4	46	—	3 0	—	4 9	2 3	—	6 5	2 7
5	42	65	2 8	1 3	3 5	2 2	3 4	4 9	5 7
6	42	12	2 8	1 5	4 3	2 3	2 7	6 0	6 4
Mean	42 5	36	2 93	1 33	4 88	2 35	3 0	5 88	4 80
SEM	1 47	17 4	0 07	0 11	0 35	0 07	0 20	0 33	0 87

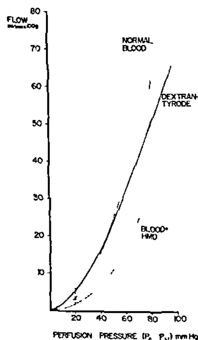


Fig 2 Pressure—flow relationships in the maximally dilated calf muscle vascular bed for dextran—Tyrode solution and normal blood (6 expts) and for the same blood when high molecular weight dextran (HMD) was added to the animal (4 expts) The height of the vertical lines on the curves at perfusion pressures 20 mm Hg and 80 mm Hg corresponds to 2 SE of the mean flow value

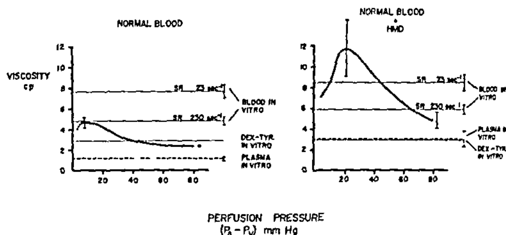


Fig 3 Average relationship between perfusion pressure (relation to flow *cf* Fig 2) and calculated viscosity for normal blood *in vivo* in 6 expts (left panel) and for blood mixed with HMD in 4 of these experiments (right panel) The calculated viscosity values *in vivo* are indicated by heavy lines Horizontal lines show values for viscosity *in vitro* for the various perfusates (for plasma dotted line), determined at shear rates (SR) 23 and 230 sec⁻¹ Vertical bars indicate 2 SE of the mean values

this possibility, plethysmographic recordings of tissue volume changes at low perfusion pressure levels were made The results of these studies indicated that at perfusion pressures below 10—15 mm Hg, tissue fluid absorption could provide, as a maximum, less than 30 per cent of the venous outflow In most cases the amount of tissue fluid added to the perfusate was negligible as indicated by the fact that the preparation remained almost isovolumetric even at very low perfusion pressures Whatever the reason is for this fluid equilibrium across the capillaries even at presumably very low capillary hydrostatic pressures, it is obvious that fluid absorption only exceptionally influenced venous outflow to an appreciable extent Therefore even at the very lowest perfusion pressures, the measured flow has been assumed to be accurate enough to utilize the lowest part of the pressure-flow curves for calculation of blood viscosity *in vivo*

In the vascular bed at high perfusion pressures (Fig 2) blood flow exceeded dextran Tyrode flow indicating that blood viscosity *in vivo* was less than that for the dextran solution. When the perfusion pressure and flow were lowered, apparent blood viscosity rose being equal to that for dextran-Tyrode at flows about 18—20 ml/min × 100 g At this point the two curves intercepted and then continued to separate, indicating that blood viscosity continued to rise as pressure and flow were reduced

The magnitude of the effects are illustrated in Fig 3 (left panel) Mean values for measurements of the same blood and dextran-Tyrode viscosity *in vitro* are also shown in this figure plotted for the lowest (23 sec⁻¹) and highest (230 sec⁻¹) shear rates used Blood viscosity *in vitro* at the highest shear rates was 4 88 cP ± 0.35 (S E of mean), more than twice the calculated viscosity *in vivo* at high flow levels present at a perfusion pressure of 80 mm Hg *i e* 2 35 cP ± 0 07 (S E of mean)

With lower pressures and flows, viscosity *in vivo* rose steadily and reached a maximum value at a perfusion pressure of 10 mm Hg, where flow was only 3 per cent of flow at 80 mm Hg. However, calculated *in vivo* viscosity was only 4.73 cP ± 0.55 (S.E. of mean) or barely the figure obtained at the *highest* shear rates *in vitro* (see above). At perfusion pressures below 10 mm Hg, when blood flow was very low, viscosity *in vivo* tended to fall rather than rise, in contrast to what might be expected from viscosity measurements made *in vitro* at low shear rates. The findings indicate that in the vascular bed under study, even at the lowest flow rates, the apparent viscosity *in vivo* would never exceed the values obtained *in vitro*, even at the *highest* shear rates used in vitro.

In the cone plate viscometer there was considerable spread of values for blood viscosity between animals. There was also moderate variation in haematocrit and especially, in erythrocyte sedimentation rate. Despite these variations, the calculated viscosity *in vivo* was remarkably similar for the same bloods, indicating that these variations in 'normal' blood from outwardly healthy cats do not significantly affect its fluid properties *in vivo* (Table I).

When, however, HMD was mixed with blood a different situation appeared (Fig. 3, right panel). At perfusion pressures above 70 mm Hg blood viscosity *in vivo* was below the *in vitro* values determined at high shear rates but at lower perfusion pressures the calculated viscosity *in vivo* rose steeply to very high values even exceeding those measured at the *lowest* shear rates used in the viscometer. However, at the lowest pressure and flow levels recorded in the vascular bed, the calculated *in vivo* viscosity declined again in much the same way as seen in the experiments with normal blood.

The rather moderate rise of blood viscosity *in vivo* at high flow rates following addition of HMD appeared related mainly to the increase of plasma viscosity. However, the greatly exaggerated increases in apparent viscosity at lower flow levels cannot be ascribed to the raised plasma viscosity *per se*. It rather suggests a disturbed suspension stability of blood and cellular aggregation, which secondarily may have caused obstruction of microvessels. Such aggregation and plugging would be facilitated by very low flow rates.

To test the possibility of capillary obstruction when the vascular bed was perfused with blood mixed with HMD at low flow rates repeated measurements of the capillary filtration coefficient (CFC) were made with the vascular bed perfused with normal blood, on the one hand and with blood mixed with HMD on the other, at both high and low flow rates. It was assumed that a significant capillary plugging should show up as a reduction of CFC. — The results from 3 experiments are shown in Fig. 4. It shows that perfusion of the maximally dilated vascular bed with normal blood at greatly varying flow rates did not appreciably affect the CFC value. CFC was also unaffected when blood mixed with HMD was perfused at high flow rates. However, if HMD blood was perfused at low flow rates a significant reduction of CFC occurred. This latter finding suggests that a considerable plugging of capillaries does take place in this situation probably as a result of aggregation

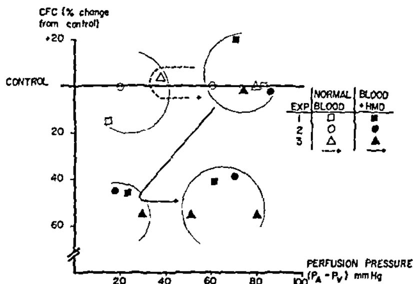


Fig 4 Alterations of CFC with changes in perfusion pressure in the maximally dilated calf muscle (open symbols) an with blood mixed with HMD re obtained during perfusion with normal blood a was no significant change of CFC when perfusi l subsequently elevated again Addition of HMD to the blood did not affect the CFC values as long as flow rates were kept high but during a period of reduced pressure and flow CFC declined markedly and remained low even when pressure and flow were subsequently elevated to control levels — The data show the results from 3 expts Each symbol represents the mean from 2—4 determinations

of red cells Such aggregation apparently does not take place when HMD blood is steadily perfused at higher flow rates as then CFC remained unchanged — However once a definite plugging was established with time by perfusion at low flow rates it was not possible to flush out aggregates by increasing flow and CFC then remained low for the rest of the experiment

Discussion

Methodological considerations Blood viscosity is regarded as an important determinant of peripheral blood flow in situations where flow rates are low such as occurs in circulatory shock. In this situation blood viscosity has been proposed to rise to such high levels as to seriously impede blood flow and tissue nutrition This presumption is based on the well known fact that blood viscosity when measured in various types of viscometers is drastically increased at low shear rates *In vitro* findings however cannot be simply extrapolated to the situation *in vivo* because of the complex morphological design of the vascular bed For example the arterioles where the main resistance to flow occurs have dimensions favouring the appearance of the Fåhraeus Lindqvist effect with consequent reduction of apparent blood viscosity When flow is reduced in connection with vasoconstriction the vascular narrowing would thus tend to lower viscosity which may in part offset the increased viscosity caused by the lowered flow velocity *per se*

The present series of experiments are an attempt to determine blood viscosity *in vivo* over a wide range of flows in order to evaluate whether alterations of blood viscosity might be a critical factor determining blood flow at low flow rates. The maximally dilated vascular bed of the calf muscles of the cat was utilized as a biological viscometer and pressure-flow curves for blood and dextran Tyrode solution were determined with flows ranging between 0.2 to 60—65 ml/min×100 g. Since dextran Tyrode solution behaves as a Newtonian fluid, the pressure-flow curve for this perfusate becomes a physical description of the vascular bed under study, a calibration curve for the biological viscometer. — From comparisons of flow values for blood and dextran Tyrode at identical perfusion pressures and using the value for dextran Tyrode viscosity as a reference, blood viscosity *in vivo* can be calculated over the whole range of flows.

To make such deductions valid, vascular dimensions must be similar for the two perfusates at each point of comparison. Therefore, to avoid dissimilarities in vascular smooth muscle tone with the two perfusates, the muscle vascular bed was maximally dilated before each flow determination by means of supramaximal muscular exercise. Furthermore, not only perfusion pressure, but also mean distending transmural pressure

$$\left(\frac{P_A - P_V}{2}\right)$$

was controlled and kept the same for the two perfusates at identical perfusion pressures. This is necessary, since the maximally dilated vessels display a considerable distensibility in the lower pressure range, which is evident from the shape of the pressure-flow curve for dextran Tyrode (Fig. 2), confirming earlier measurements by Folkow and Löfving (1956). — Therefore, since the possibility of pressure-passive as well as active alterations in vascular dimensions were essentially eliminated, the assumption of identical vascular dimensions at a given perfusion pressure for each perfusate seems reasonable.

A further critical point in the present analysis is the accuracy of the flow measurements, particularly at the very lowest flow range. The method of direct sampling of the venous effluent over a relatively long time period in a graduated pipette gives a very accurate and reproducible result as shown in Methods. The problem whether the venous outflow really represents the blood flow through the tissue or, at very low flow rates, mainly absorbed tissue fluid has been briefly discussed earlier. Very low levels of perfusion pressure imply a considerable lowering of capillary pressure as well, and it may be argued that this leads to a substantial fluid absorption as the entire capillary network should be open to flow. However, simultaneous volume recordings indicated only a minute fluid absorption at these low pressures and flows, suggesting that either many loops of the capillary network were brought to collapse or that their content become stagnant at very low pressures. This means that the capillary surface area available for absorption becomes reduced, too. In fact, the volume recordings showed that tissue fluid absorption usually accounted for less than

10—20 per cent and as a maximum 30 per cent of the flow in the venous outflow tubing with no significant difference between blood and dextran Tyrode Furthermore, systematic measurements of CFC at different perfusion and transmural pressures in the maximally dilated calf muscle preparation show a sharp reduction of CFC when capillary pressure falls below some 15 mm Hg especially when the pressure gradient and flow are low (Folkow, Gaskell and Waaler 1969) Therefore the relationship between the flow values for blood and dextran Tyrode seems to be fairly accurate and representative even at the lowest pressure levels

Evaluation of the results The results of the present study indicate in agreement with previous studies (Whittaker and Winton 1933 Levy and Share 1953) that blood viscosity *in vivo* at high flow rates is normally much lower than the viscosity *in vitro*, even when the latter is measured at high shear rates In addition the present results show that normal blood viscosity *in vivo* increases at reduced flow but even at very low flow rates it barely reached the minimal *in vitro* values found at high shear rates This finding is of considerable significance, since it indicates that the presence of red cells *per se* would not be an important flow limiting factor in the vascular bed when flow velocity is reduced to very low levels at least as long as blood composition is normal It may be speculated that the reason for this is that as perfusion pressure falls and the vascular bed exhibits elastic recoil blood moves through a vascular bed where a more extended portion of the resistance vessels have now become so narrow that the cells move in a few or even in a single file (bolus flow) If so this would imply a *reduced* viscosity in such vessels offering high resistance to flow while in wider tubes of less importance for total resistance, viscosity would increase because of the reduced flow rates.

That indeed a marked reduction of vessel dimensions occurs with lowering of the perfusion pressure is evident from the convexity of the pressure-flow curves If the vascular bed could be replaced by a hypothetical rigid tube the pressure flow relation would be represented by a straight line approximately running from the highest point of the dextran Tyrode curve to the origin (Fig 2 The passive reduction of the radius of an idealized vessel due to elastic recoil can then be deduced for each perfusion pressure by comparing the actual dextran Tyrode flow with the flow in the hypothetical rigid system Such deductions make it clear that the mean radius of the idealized vessel decreases progressively with perfusion pressure At a perfusion pressure of 10 mm Hg the radius has decreased by approximately 30 per cent compared with that at 80 mm Hg

A blood viscosity of 2 35 CP *in vivo* as observed in this study at high rates of flow, corresponds largely to values observed *in vitro* when blood is perfused through tubes of approximately 30—40 μ diameter (Bayliss 1952 This suggests that in the present vascular bed the average diameter of the resistance vessels is of this order of magnitude, *i e* that approximately half of the resistance resides in tubes of still smaller dimensions (metarterioles capillaries etc) A recoil corresponding to 30 per cent decrease of diameter in such vessels would mean that an increased portion of them now become so narrowed that bolus flow emerges It is known that blood

viscosity in such narrow vessels is rather close to that of plasma (Bayliss 1952 Prothero and Burton 1962) and the mentioned changes in vascular dimensions must therefore affect apparent blood viscosity

One should for such reasons expect an even more extensive depression of apparent blood viscosity in shock where vascular dimensions due to neurogenic vasoconstriction are decidedly smaller than in the maximally dilated state and therefore linear flow rates correspondingly higher for a given blood flow A hypothetical example may be visualized thus Suppose a shock situation exists with a muscle blood flow as low as 0 5 ml/min × 100 g at a perfusion pressure of 60 mm Hg The same flow is produced by a pressure head of only some 5 mm Hg in the maximally dilated bed used in the present study This implies that regional flow resistance is 12 times higher in the shock situation, *i e* the mean radius of an idealized resistance vessel is then only 55 per cent of radius in the maximally vasodilated state Here a much larger portion of the resistance vessels must be narrow enough to enforce cell passage in a few or even a single file

Moreover in the above example average linear flow velocity is about 3 times higher in the constricted state for a given blood flow This factor will further tend to reduce apparent blood viscosity Therefore as long as the physical properties of blood *i e* suspension stability etc are not disturbed blood viscosity *in vivo* probably never reaches values high enough to appreciably affect blood flow It should be stressed that blood viscosity was not more than doubled at the extremely low flow velocities studied in the maximally dilated vascular bed The range of viscosity change met with normally or even in shock must then be considerably smaller as long as blood composition remains the same

At extremely low perfusion pressures and flows (below about 5 mm Hg) blood viscosity *in vivo* showed no tendency to increase further in some experiments it even decreased Calculations of blood viscosity from the very lowest part of the pressure flow curves are admittedly given to error unless accurate methods are used to determine the very low pressure gradients and blood flows However all precautions were taken to achieve exact data and the finding suggesting a fall of blood viscosity might therefore very well represent a *true* reduction of viscosity *in vivo* at very small flows and vascular dimensions One explanation might be the escalated capillary closure which seems to occur at very low pressures and flows (Folkow Gaskell and Waaler 1969) Capillary flow resistance must correspondingly increase its contribution to *total* flow resistance in such situations This again means that the very low blood viscosity in these narrow tubes becomes more important for apparent blood viscosity as well which consequently may decrease slightly

The relatively high values for blood viscosity obtained in various types of laboratory viscometers can probably be explained by the absence of the Fahraeus Lindqvist effect and of the dynamic changes that occur in the vascular bed as tube dimensions and flow velocity alter in the complex manner outlined above However it cannot be excluded that some of the characteristic *in vitro* findings may be due to an artefact in terms of physical changes in the cell fraction as a consequence of

extraneous factors, such as contact with glass and metal Such factors might for example explain why the viscosity values, measured *in vitro* for blood taken from different animals showed a rather wide variation unrelated to hematocrit erythrocyte sedimentation rate and plasma viscosity, while the *in vivo* values for the same blood samples were essentially identical (Table I) No attempts were made to analyze whether the *in vivo* values also became more scattered if the blood was taken out from the animal and for example, brought in contact with glass before it was perfused through the preparation Some aspects of such a comparison may be illustrated by the study by Skovborg Nielsen and Schlichtkrull (1968) where human blood was perfused through the isolated rabbit s ear in comparison to saline Only fairly high perfusion pressures were studied but approximate proportionality between *in vivo* and *in vitro* values were observed in this study — Whatever the reasons are for the considerable variations in *in vitro* viscosity of cat s blood it is clear that blood showing a high viscosity value *in vitro* may very well exhibit a quite low viscosity *in vivo* For such reasons one is inclined to wonder to what an extent measurements of blood viscosity in standard viscometers may be misleading for evaluations of the biological situations *i e* when blood is flowing in the vascular bed which no doubt is the place where its viscosity is really relevant

Pathophysiological aspects While the viscosity for *normal* blood *in vivo* never seems to rise to such high levels as to considerably affect flow the situation becomes quite different if the blood is mixed with high molecular weight dextran (HMD) which is known to alter the physical properties of blood in such a way as to disturb the erythrocyte suspension stability and promote cell aggregation (*e g* Gelin Rudenstam and Zederfeldt 1965 a) Addition of HMD to the blood in the amounts used in the present experiments produced moderate rises of plasma and blood viscosity *in vitro* and also a rise of blood viscosity *in vivo* at high perfusion pressures and flows The most striking effect of adding HMD to blood was however the marked elevation of the viscosity *in vivo* at lower flow rates to values well above even the *maximum* levels found *in vitro* at the lowest shear rate used This is in sharp contrast to the behaviour of *normal* blood where the highest *in vivo*-values when averaged were below the *lowest* viscosity values determined *in vitro* at high shear rates

This disproportionate increase of viscosity *in vivo* of cell containing fluid after addition of HMD suggests that physical changes in the red cells occur at low flow rates leading to the formation of red cell aggregates The formation of such aggregates could result in the obstruction of small vessels and hence affect also the calculated values for viscosity That such a plugging of small vessels in fact takes place is supported by the present finding of a markedly reduced number of perfused capillaries as indicated by a decreased CFC when the vascular bed was perfused with blood mixed with HMD at low perfusion pressures (Fig 4) The appearance of this obstruction of small vessels coincides with the dramatic increase in the calculated viscosity *in vivo*

As long as only higher flow rates were used—where the viscosity *in vivo* is relative

ly low and the movement of the blood would tend to prevent formation of aggregates —determinations of CFC gave no indication for capillary plugging and derangement of the microcirculation It therefore seems reasonable to assume that the effects of HMD on calculated blood viscosity *in vivo* are mainly related to the effects of cell aggregation precipitated when very low flow rates occur Therefore the high viscosity values at low flow rates are at least in part spurious and caused by vessel obstruction and altered architecture of the microvascular network rather than due to a genuine rise of blood viscosity *per se* This would also explain the significantly lower *in vitro* viscosity values at low shear rates as compared with *in vivo* figures at low flows since cell aggregation would obviously not affect a laboratory viscosimeter in the same way Thus even if the *in vitro* values are in this very respect more representative than *in vivo* values for the *genuine* viscosity of blood mixed with HMD they do not reveal the serious complications that can occur when blood is flowing in the vascular bed

An increased tendency for cell aggregation might be expected to facilitate the appearance of bolus flow also at larger vessel dimensions This might partly explain the observed decrease in viscosity *in vivo* for blood mixed with HMD at very low flow rates as was sometimes also seen with normal blood This decrease of *in vivo* viscosity occurred at slightly higher pressures and hence vascular dimensions in the HMD experiments than with normal blood

It should be noted that the present study predominantly deals with the influence of blood viscosity factors on the magnitude of volume flow through the vascular bed and to a lesser extent on the *distribution* of this flow over the capillary network as determined by CFC measurements Normal blood viscosity *in vivo* never seems to rise to levels that might seriously impair either one of these two functions However still another aspect of blood viscosity *in vivo* should be considered It is possible that with extremely low flow rates local viscosity rises significantly more in the wide bore venous section than in the precapillary section Although such a relatively more pronounced increase of regional blood viscosity in the veins will not significantly affect *total* hindrance to flow it may result in a considerable rise of the low postcapillary flow resistance This would other factors remaining constant lower the pre to postcapillary resistance ratio and cause an elevation of capillary hydrostatic pressure In critical situations such a shift might lead to an impeded fluid absorption at the capillary level or even to filtration losses from the circulatory system and a decreased plasma volume (Mellander and Lewis 1963) Thus considerable disturbances of normal function of the vascular bed might be caused by such subtle alterations of local blood viscosity that cannot be detected by the present experimental approach

Serious derangements of the peripheral circulation resulting from altered properties of the streaming blood are no doubt mainly met with under conditions of increased cell aggregation The present results suggest that the most serious consequence may be related to obstruction of smaller vessels by cell aggregates rather than to increased blood viscosity *per se* Clinical observations (*e g* Gelin 1956) point in

the same direction Such a situation, which has been experimentally produced in the present experiments by addition of HMD appears to occur in traumatic and burn shock due to greatly reduced peripheral flow rates as combined with disturbances in the suspension stability following a changed plasma protein composition (Gelin, Rudenstam and Zederfeldt 1965 b) Especially the increased fibrinogen concentration seems to be important also *in vitro* greatly increasing blood viscosity (Chien *et al* 1967) If poor perfusion proceeds for long periods, aggregates probably settle in the microcirculation and may then often resist the flushing action of sudden elevations of perfusion pressure leading to a more permanent derangement of the microcirculation to judge from the present study (Fig 4)

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The CSF/Blood Potential in Sustained Acid-Base Changes in the Rat. With Calculations of Electrochemical Potential Differences for H^+ and HCO_3^-

By

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Received 17 March 1969

Abstract

KJALLQUIST, A *The CSF/blood potential in sustained acid/base changes in the rat With calculations of electrochemical potential differences for H and HCO_3* Acta physiol scand 1970 78 85—93

The electrical D C potential difference between cisternal cerebrospinal fluid and external *jugular blood was measured in rats during sustained acid base changes One group, serving as* [illegible] other groups were [illegible] 3 solutions In the [illegible] ly administered so [illegible] groups the CSF/plasma potential was permanently changed in relation to the arterial plasma pH but there was a larger potential change in alkalosis (about 50 mV/pH unit) than in acidosis (about 30 mV/pH unit) Calculations of electrochemical potential differences for H^+ and HCO_3 between CSF and plasma showed no significant differences in nonrespiratory acidosis and alkalosis but significant increases in hypercapnia The results do not support the theory that an active H^+ transport regulates the CSF pH to constancy

After that Tschirgi and Taylor (1954, 1958) demonstrated the existence of an electrical D C potential difference between subarachnoidal fluid and jugular blood in the rat, the potential difference between cerebral extracellular fluids and extra neural tissue has been studied repeatedly in various experimental animals These studies have found ventricular or cisternal CSF to be 1—7 mV positive referred to blood (Held, Fencl and Pappenheimer 1964, Severinghaus *et al* 1963 Mitchell *et al* 1964), or to extradural tissues (Motschall and Loeschke 1963)

Tschirgi and Taylor (1954, 1958) originally reported that the CSF/blod potential difference (P D) was sensitive to both the hydrogen ion and potassium ion concentrations of the plasma *(cf* Kao and Loeschke 1965) but later studies by Held *et al* (1964) showed that only the hydrogen ions changed the P D from the blood side, while in the CSF only the potassium ion concentration had an effect, although

slight, on the potential These authors found a linear inverse relation between the arterial plasma pH and the CSF/blood P D in dogs and goats with a slope of 32 and 43 mV per unit change of pH in acute respiratory and nonrespiratory acid base changes respectively

It has been pointed out (Severinghaus *et al* 1963, Held *et al* 1964) that there is a net electrochemical potential difference for hydrogen ions between CSF and blood plasma With a CSF/plasma P D of +4 mV, and at CSF and plasma pH values of 7 33 and 7 38 respectively, the $\Delta\mu H^+$ can be calculated to about 7 mV according to the equation

$$\Delta\mu H^+ = 61\ 5\ (pH_{pl} - pH_{CSF}) + \Psi$$

where Ψ is the P D between CSF and plasma (Held *et al* 1964 Severinghaus 1965 see also Kjällquist and Siesjö 1968 Siesjö and Kjällquist 1968)

The existence of a net $\Delta\mu H^+$ has been interpreted to indicate an active transport of H between plasma and CSF (see Severinghaus *et al* 1963, Held *et al* 1964 Severinghaus 1965) The assumption of an active transport regulation of the CSF pH seemed to be supported by the constancy of the CSF pH in a variety of acid base changes (see Mitchell *et al* 1965) and by the $\Delta\mu H$ values calculated by Severinghaus (1965) on the assumption that the CSF/plasma P D returned to normal in chronic acid base changes However recent measurements (Goodrich 1965 Kjällquist and Siesjö 1967 1968) have shown that the P D changes are upheld in chronic experimental acid base changes In view of these results the $\Delta\mu H$ values between CSF and plasma and their implications for the interpretation of the CSF pH regulation must be re evaluated (see Siesjö and Kjällquist 1969)

The present report describes measurements of the CSF/blood P D in the rat during sustained nonrespiratory and respiratory acid base changes From these potential measurements and from known pH and bicarbonate differences electrochemical potential differences for H and HCO_3^- could be calculated The results do not corroborate the hypothesis of an active transport regulation of the CSF pH

Methods

The present results were obtained on male Wistar rats weighing 300—450 g There were 5 groups of rats Three of these groups were injected i p every second hr with either a blank solution a NH_4Cl solution or a $NaHCO_3$ solution (Siesjö and Pontén 1966 a) The remaining two groups were either given 60 ml/kg of a NH_4Cl solution or 100 ml/kg of a $NaHCO_3$ solution i p in three doses during 6 hrs However in the NH_4Cl group 10 % CO_2 was administered throughout so as to give a normal plasma bicarbonate concentration and in the $NaHCO_3$ group 7 % CO_2 was given to keep the plasma pH constant (Siesjö and Pontén 1966 b)

At the end of the 6 hrs equilibration period the animals were anesthetized with phenobarbital (150 mg/kg i p) and tracheotomized During the performance of the tracheotomy the hypercapnic rats were given the CO_2-containing gas mixture through a rubber mask One femoral or tail artery was then cannulated for blood pressure recording with an electromanometer (Elema Stockholm) and for sampling of arterial blood The rats were placed on an electrically grounded metal box but they were isolated from the box by means of a plastic sheet and their heads were fixed with plastic earpins The body temperature was measured rectally with a mercury thermometer

Arterial blood was analysed at 37° C for pCO_2 pO_2 and pH using microelectrodes (Eschweiler, Kiel and Radiometer Copenhagen) The pH values were referred to the equimolar

phosphate buffer of the N B S (pH=6 841 at 37° C) All values were corrected for differences in temperature between the electrodes and the animals The actual bicarbonate concentrations and the base excess values were obtained from the nomogram of Siggaard-Andersen (1963).

The CSF/plasma potential difference

The P.D was measured between the external jugular vein and the cisterna magna, using bridges of 3 M KCL in 2 5 % agar, connected to saturated KCl-calomel electrodes (type K 100, Radiometer, Copenhagen) The calomel electrodes were connected to a differential amplifier with an input impedance of 2×10^{10} Ω In most instances, the P D was recorded continuously on a potentiometric recorder The external jugular vein was cannulated with a polyethylene tubing (I D = 0 6 mm) containing the KCl-agar gel The cisterna magna was punctured with a borosilicate glass pipette, which was filled with the same gel, and mounted ... was exposed in a dry ... e first part of the in- ... 3S −1, Ethicon), and ... the last part of the ... ighly dried membrane

The asymmetry potential between the plastic tubing and the glass pipette was measured in Krebs-Henseleit solution before and after each *in vivo* measurement Only initial asymmetry potentials of less than 0 5 mV were accepted, and if the asymmetry potential changed by more than 1 5 mV during the experiment, the experiment was discarded Within the latter ... ial during the experiment, ... ts the P D was measured ... oral artery The P.D did

The criteria for a successful punction were as follows When the glass pipette was placed on the membrane with a slight pressure an electrostatically sensitive potential difference of −8 to −15 mV was measured At the puncture the P D jumped suddenly to a higher and ...

not satisfied were excluded from the material

Calculations of the electrochemical potential differences

The electrochemical potential differences for HCO_3^- and H^+ between CSF and plasma were calculated from the present potential measurements and from the CSF/plasma H^+ and HCO_3^- relations previously published from the laboratory (Siesjö and Ponten 1966 a and b) The strict relationship between the plasma pH and the CSF/plasma potential obtained in the present experiments made it possible to obtain from the curve of Fig 1 the appropriate CSF/plasma potential for the previously published rat groups However, a recalculation was made from the individual experiments of those groups Thus, the actual bicarbonate concentration of arterial plasma was calculated from the pH and pCO_2 values, using a pK which was corrected for temperature and pH, and a solubility coefficient which was corrected for temperature (Severinghaus, Stupfel and Bradley 1956) The mean capillary bicarbonate concentration was then calculated for capillary plasma water (0 935 g/ml of plasma), assuming a pCO_2 dependent linear relation between the arterial and the mean capillary bicarbonate concentrations Thus, the difference between the mean capillary and the arterial plasma bi... 1 1 meq/l at a pCO_2 of 40 mm Hg, and 0 6 ... Kety and Smith 1948, own unpublished results) ... derived from an assumed linear relation with a ... Hg and a difference of 0 01 at a pCO_2 of 70 mm Hg

Results

The results of the P D measurements are shown in Table I and in Fig 1 Fig 1 shows the relation between the plasma pH and the CSF/plasma potential in the individual rats It is seen that the relation between the change in pH and the change in potential was the same irrespective of the mode of altering the pH. Table I gives

TABLE I Acid base parameters in arterial plasma and CSF/blood potentials in the rat groups studied (see Methods and Fig 1) The bicarbonate concentrations and the base excess values were calculated from the pCO_2 and the pH values Figures within parentheses denote number of experiments The values are means ± S E M

Group	Arterial plasma				CSF/blood P D mV
	PCO_2 mm Hg	HCO_3^- meq/l	Base Excess meq/l	pH	
Blank (7)	35 7±1 4	22 3±1 3	− 1 1±1 3	7 413±0 017	+ 2 8±0 6
$NaHCO_3$ (5)	43 8±3 3	35 3±2 8	+11 3±2 2	7 520±0 007	− 1 6±0 5
NH_4Cl (5)	36 8±1 4	16 3±0 6	− 9 4±0 9	7 274±0 022	+ 8 7±0 7
$NaHCO_3+CO_2$ (5)	60 0±1 2	36 4±1 0	+10 0±0 8	7 400±0 005	+ 4 6±0 4
NH_4Cl+CO_2 (5)	65 6±3 3	23 8±1 1	− 5 6±1 0	7 188±0 018	+10 9±0 4

the mean values for the blood parameters and for the CSF/plasma potential in the five groups studied

The results quite clearly show that a "chronic" change in plasma pH was associated with an altered CSF/blood P D However, the slope of the curve ($\Delta\Psi/\Delta pH$) was different in acidosis and in alkalosis Thus, the mean slope in the acid direction was about 30 mV/pH unit and about 50 mV/pH unit in the alkaline direction

Table II lists the mean capillary and the CSF bicarbonate concentrations and pH values calculated from the rat groups of Siesjo and Pontén (1965, 1966), together

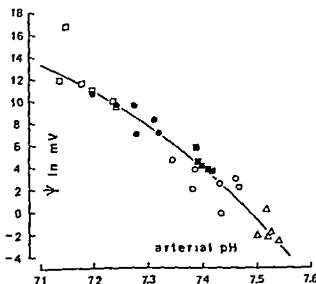


Fig 1 Relation between arterial plasma pH and the CSF/plasma potential (ψ) of rats made acidotic (filled circles) or alkalotic (triangles) by means of i p injections of NH_4Cl or $NaHCO_3$ The control group (unfilled circles) was given a simulated extracellular fluid i p In the two last groups, $NaHCO_3$ or NH_4Cl solutions were given but CO_2 was simultaneously administered to keep either the plasma pH (filled squares) or the plasma bicarbonate (unfilled squares) constant. The curve was obtained by best visual fit.

TABLE II Calculations of electrochemical potential differences for H^+ ($\Delta\mu H^+$) and for HCO_3^- ($\Delta\mu HCO_3^-$). The arterial and CSF acid base values were taken from two previous publications from the laboratory (Siesjö and Pontén 1965, 1966), while the corresponding values were derived from Fig. 1. The arterial and CSF values were recalculated as described in the text (see Methods). Number of experiments within parenthesis. The values are means ± S.E.M. The standard errors given for the $\Delta\mu H^+$ and $\Delta\mu HCO_3^-$ values are minimal values since they were calculated on the assumption of a single relation between arterial pH and Ψ.

Group	Arterial plasma		Capillary plasma water		CSF		Ψ mV	$\Delta\mu H^+$ mV	$\Delta\mu HCO_3$ mV
	pCO_2 mm Hg	pH	HCO_3^- meq/l	pH	HCO_3^- meq/l	pH			
Blank	37.3	7.44	28.5	7.41	28.3	7.43	+2.2	+0.7	−2.0
(6)	±1.3	±0.00	±1.0	±0.00	±0.6	±0.01	±0.2	±0.4	±0.8
$NaHCO_3$	42.3	7.55	40.4	7.52	32.0	7.45	−3.3	+1.1	−2.9
(6)	±0.9	±0.01	±0.7	±0.01	±0.5	±0.01	±0.7	±0.5	±0.5
NH_4Cl	35.9	7.28	18.8	7.25	24.5	7.39	+8.4	−0.2	−1.2
(6)	+1.6	±0.02	±1.0	±0.02	±0.7	±0.01	±0.7	±0.9	±0.9
Blank + CO_2	55.9	7.35	33.4	7.33	33.0	7.36	+6.0	+4.5	−6.3
(6)	±1.7	±0.01	±0.9	±0.01	±0.4	±0.01	±0.5	±0.8	±0.7
$NaHCO_3$ +	50.2	7.44	36.8	7.41	30.9	7.37	+2.6	+5.0	−7.2
4 % CO_2 (7)	±0.9	±0.00	±0.6	±0.01	±0.2	±0.01	±0.2	±0.4	±0.5
$NaHCO_3$ +	70.6	7.40	46.6	7.39	37.2	7.33	+4.1	+8.0	−10.1
7 % CO_2 (6)	±1.3	±0.01	±1.2	±0.01	±0.7	±0.01	±0.6	±0.3	±0.3
NH_4Cl +	57.0	7.29	29.5	7.27	31.2	7.32	+8.3	+4.8	−6.4
7 % CO_2 (5)	±0.5	±0.02	±1.0	±0.02	±0.4	±0.00	±0.6	±0.6	±0.6
NH_4Cl +	76.3	7.19	30.5	7.19	34.4	7.27	+11.2	+6.2	−7.4
10 % CO_2 (4)	±0.8	±0.01	±1.3	±0.01	±0.5	±0.01	±0.3	±0.7	±0.2

with the P.D. values derived from Fig. 1, and the calculated electrochemical potential differences. The table shows that there were no significant differences in the $\Delta\mu H$ and $\Delta\mu HCO_3$ values between the normocapnic groups, but that there were increases in the electrochemical potential differences in all the hypercapnic groups. The $\Delta\mu H$ and the $\Delta\mu HCO_3$ values calculated were obviously closely related to the CO_2 tension (Fig. 2), but unrelated to the CSF pH (Fig. 3), and to the arterial pH or bicarbonate concentration.

Discussion

There is now a large number of reports of measurements of the potential between CSF and blood, or of the potential across the plexus chorioid epithelium (for literature references see Held *et al.* 1964). Most of these studies have been devoted to

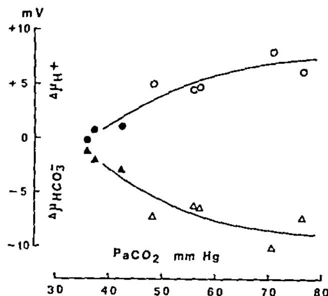


Fig 2 Relation between the calculated electrochemical potential differences for H^+ ($\Delta\mu H$, circles) and HCO_3 ($\Delta\mu HCO_3$ triangles) between CSF and plasma and the arterial CO_2 tension The normocapnic groups are denoted by filled symbols (see Table II) Note increasing $\Delta\mu$ values at increasing CO_2 tensions

the influence of various ions and of metabolic inhibitors on the potentials In some of the studies the potential has been measured in *in vitro* situations which are somewhat remote from the physiological ones, and in others, the electrodes have been placed in fluids, the ionic composition of which are unknown Since our interest concerns the relation between the potential and the distribution of ions between the CSF and blood plasma we will limit our main discussion to those studies in which both the potential and the pH or the bicarbonate concentrations of CSF and plasma have been measured

) The CSF is 3—5 mV positive to blood at a plasma pH of about 7 4 in both goats dogs and rats (Held *et al* 1964 Severinghaus 1965, Kjallquist and Siesjo 1967) In

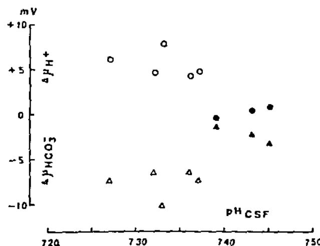


Fig 3 Relation between the calculated electrochemical potential differences for H^+ ($\Delta\mu H$) and HCO_3 ($\Delta\mu HCO_3$) between CSF and plasma and the CSF pH (*cf* Fig 2) There is no simple relation between the calculated $\Delta\mu$ values and the CSF pH values

these animals the potential changes to a more positive value in acute respiratory and nonrespiratory acidosis (*cf* however, results on cats reported by Besson and Marshall 1968), while the CSF side gets more negative in the corresponding alkalotic conditions This shows that the potential is altered by the plasma pH and not by the CO_2 tension, a conclusion which is amply supported by the present experiments

Although the potential is altered by changes in plasma pH, the potential is not a simple H potential in the sense that it is set by the H^+ distribution between CSF and plasma The fact that the potential is influenced by the potassium concentration of the CSF (Held *et al* 1964) indicates that the membrane systems separating CSF and plasma are *functionally similar to frog skin with its asymmetric sodium/potassium sensitivity* (Ussing 1953) However, although the mechanisms which determine the potential are unknown, we may discuss and evaluate electrochemical potential differences for H^+ and HCO_3 as long as the potential and the concentrations of the ions are measured in the same phases

The absolute electrochemical potential differences calculated are approximations the exactness of which depend on the validity of the assumptions which are inherent in the calculation Thus, when we apply the above equation (see Introduction) it is assumed that there are no appreciable hydrostatic pressure gradients, and no significant solvent drag effect (Ussing 1965) Likewise, both the electrical potential difference and the pH difference measured will include any difference in junction potential between CSF and blood Further, when bicarbonate concentrations in blood and the pH values in CSF, are calculated errors in the pK′ values used [illegible] add to the uncertainty of the $\Delta\mu$ values derived Finally, there is no exact way of estimating mean capillary pH values and bicarbonate concentrations from the measured plasma values The possible errors of the assumptions cast some doubts on the absolute values for the $\Delta\mu H$ values, although the $\Delta\mu H^+$ value of about 7 mV calculated for control conditions in dogs and goats (Severinghaus *et al* [illegible] Held *et al* 1964) probably is much too large to be accounted for by errors in the [illegible] tions However, such errors probably explain why the $\Delta\mu HCO_3$ and $\Delta\mu$[illegible] in the present materials do not add up to zero In the rat, the CSF bicarb[illegible] high that the $\Delta\mu H$ calculated at a plasma pH of 7 4 is close to zero, but [illegible] obtained in chronic acidosis show that an appreciable $\Delta\mu H$ can be [illegible] this animal

The errors inherent in the above assumptions makes it safer to eval[illegible] in $\Delta\mu H$, or in $\Delta\mu HCO_3$, than absolute values It has been [illegible] that the absolute $\Delta\mu H$ found in control conditions (Severinghaus [illegible] Held *et al* 1964) indicates active transport of H from plasma to CSF, [illegible] this active transport of H regulates the CSF pH to constancy [illegible] transport hypothesis was reported to be supported by $\Delta\mu H$ values cal[illegible] acid base changes in the dog (Severinghaus 1965) but it was [illegible] CSF/plasma potential returned to normal in the chronic state

The present results have shown that the changes in the [illegible] with the plasma pH are upheld in chronic acid base cond[illegible]

1965, Kjallquist and Siesjo 1967, 1968) They have also shown that the calculated $\Delta\mu H$ and $\Delta\mu HCO_3$ values are not significantly different in metabolic acidosis and alkalosis and that they are significantly increased in all hypercapnic conditions In fact the electrochemical potential differences appear unrelated to the plasma or CSF pH, to the pH difference between CSF and plasma or to the bicarbonate concentrations but they are closely related to the CO_2 tension (see Fig 2) What is the implication of the calculated $\Delta\mu$ values for the hypothesis of an active H transport from plasma to CSF regulating CSF pH? If the active transport regulates the CSF pH to constancy we would expect a reduced rate of transport in all conditions in which there is a tendency for the CSF pH to change in the acid direction and an accelerated rate of transport in the corresponding alkalotic conditions But if the H transport causes the net $\Delta\mu H$ between the CSF and the plasma this is to say that we would expect $\Delta\mu H$ to decrease in acidosis and increase in alkalosis Obviously the results obtained in the present experiments do not corrobate the active transport hypothesis referred to above Thus although the $\Delta\mu H$ values obtained in the hypercapnic conditions support the general hypothesis of an active transport of H between the plasma and the CSF (*cf* however, discussion of interaction with the third system *i e* the tissue in Siesjo and Kjallquist 1968 1969) there is no indication that this active transport regulates the CSF pH to constancy Thus in non respiratory acidosis and alkalosis the $\Delta\mu H$ values are so similar that a change in the rate of transport in response to acidosis and alkalosis appears improbable Moreover in all the hypercapnic conditions the $\Delta\mu H$ is increased indicating an increased rate of H transport from plasma to CSF which would aggravate the acidosis already induced by the high CO_2 tensions

The increase in the $\Delta\mu H$ in hypercapnia already indicated by the preliminary experiments (Kjallquist and Siesjo 1968) has in combination with other results led us to set up a new hypothesis for the regulation of the CSF pH (Siesjo and Kjallquist 1969) The hypothesis assumes that the purposeful regulation of the CSF pH is the change in the CSF/plasma potential in response to a change in the plasma pH while the postulated active transport of H between plasma and CSF is assumed to regulate the intracellular pH of brain cells at the expense of the CSF pH constancy The present results have shown that the CSF/plasma potential may be an important regulator of the CSF pH It is especially impressive that the potential changes by about 50 mV/pH unit when the plasma pH is changed in the alkaline direction The results also support the theory in showing the progressive increase in the calculated $\Delta\mu$ values with increasing CO_2 tensions These results in conjunction with results showing the influence of carbonic anhydrase inhibitors upon the buffer capacity of the intracellular space (Kjallquist Messeter and Siesjo in press) strongly indicate that the postulated H transport between plasma and CSF is a transcellular transport of H which is triggered by changes in the intracellular pH of cells in the brain

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Acta physiol scand 1970 78 94—102

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The In Vivo CO_2 Buffer Capacity of Rat Brain Tissue under Carbonic Anhydrase Inhibition

By

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Received 17 March 1969

Abstract

KJÄLLQUIST A. K. MESSETER and B K. SIESJÖ *The in vivo CO_2 buffer capacity of rat brain tissue under carbonic anhydrase inhibition* Acta physiol. scand 1970 78 94—102

The effect of acetazolamide upon the *in vivo* CO_2 buffer capacity of rat brain tissue was investigated by injecting the animals with the drug in two doses of 50 mg/kg each before they were anesthetized and subsequently exposed to various CO_2 tensions for 40—60 min. Acetazolamide was found to give a marked lowering of the CO_2 buffer capacity Thus the intracellular buffer capacity calculated as $\beta = \Delta \log pCO_2 / \Delta pH$ was found to be about 1.4 and thus considerably lower than the intracellular CO_2 buffer capacity of animals not given acetazolamide but otherwise treated in an identical way ($\beta = 2.3$ see Kjällquist Nardini and Siesjö 1969 a The results indicate that acetazolamide interferes with an active transport of hydrogen ions between cells and the extracellular fluids which is dependent on carbonic anhydrase

It has recently been shown Kjällquist, Nardini and Siesjö 1969 a that if rats anesthetized with nitrous oxide are exposed to different CO_2 tensions for 40—60 min. the calculated intracellular buffer capacity is higher than that previously reported for rats anesthetized with phenobarbital (Pontén 1966) The results suggest that the buffer capacity to CO_2 i e the change in the equivalent intracellular pH (pH' see Kjällquist Nardini and Siesjö 1969 a) for a given change in CO_2 tension *is influenced by the anesthetic used possibly through an effect on the metabolism.* Such a conclusion is not without theoretical support since it has been pointed out that if the intracellular bulk pH in muscle and nerve cell is around 7 metabolic energy must be used to extrude hydrogen ions against the electrochemical gradient (see *e g* Caldwell 1956 However this metabolically linked extrusion of hydrogen ions has remained an enigma since there have been no suggestions as to the coupling between the rate of metabolism and the rate of proton flux or about any triggering mechanisms involved

We have recently pointed out that the assumed active transport of hydrogen ions from plasma to cerebrospinal fluid varies directly with the CO_2 tension, and that it is inhibited by carbonic anhydrase inhibitors like acetazolamide (Siesjö and Kjällquist 1969, Kjällquist 1969). The characteristics of this assumed transport suggests that it reflects the activity of a hydrogen ion transferring pump in carbonic anhydrase containing brain cells, purposefully regulating the pH in those cells when the CO_2 tension varies (Siesjö and Kjällquist 1969). However, if such a mechanism is catalyzed by carbonic anhydrase it should be inhibited by acetazolamide and, consequently, a lower CO_2 buffer capacity should be expected when the tissue carbonic anhydrase is inhibited.

The present experiments were devised to study the effect of carbonic anhydrase inhibition upon the CO_2 buffer capacity of brain tissue *in vivo*. To that end rats were injected with acetazolamide in two doses of 50 mg/kg each and subsequently exposed to different CO_2 tensions for 40—60 min. At the end of the exposure time, CSF and brain tissue were sampled for analysis of bicarbonate. Preliminary experiments were made to assess the mean tissue CO_2 tension at the various arterial CO_2 tensions encountered.

Methods

The experimen[illegible]
injected [illegible]
at a 0 [illegible]
[illegible] was cannulated for blood pres[illegible] The atlanto-occipital membrane [illegible]eans of reflection of the neck [illegible]t and a plastic funnel was fitted into [illegible] with liquid nitrogen (Ponten 1966) [illegible] arterial blood at 37° C using micro[illegible]nd Co Kiel) and the haemoglobin
was mea[illegible]
and Sies[illegible]
analysed [illegible]
tentorial parts of the brain tissue were analysed for the total CO_2 content and for the water content (Pontén and Siesjö 1964).

The arterial (and tissue) CO_2 tension was lowered by increasing the volume of inspiration and increased by adding CO_2 to the gas mixture delivered to the respirator. Five to ten min after the change in the ventilation or after giving CO_2 the arterial pCO_2 was measured. The CO_2 tension obtained was then upheld for 40—60 min after which time CSF was sampled and the brain tissue was frozen. Before the sampling two measurements were made of the arterial CO_2 tension with an interval of about 10 min. After sampling of CSF a fourth blood sample was usually taken for measurement of the arterial CO_2 tension.

In the first part of the investigation the relation between the measured (apparent) arterial CO_2 tension and the CSF CO_2 tension was studied. The comparison was made between the CO_2 tension measured in the CSF and the mean value of the arterial CO_2 tensions measured before and after the sampling of CSF. For measurement of the CSF CO_2 tension the gas mixture used for calibrating the CO_2 electrodes was kept as close as possible to the expected CO_2 tension (Ponten and Siesjo 1966, se also Brzezinski *et al.* 1967).

In the second part of the investigation a study was made of the relation between the tissue CO_2 tension equated with the CSF CO_2 tension (see Fig. 1) and the CSF and tissue bicarbonate concentrations. The CSF bicarbonate concentration was derived by subtracting from the total CO_2 content the amount of CO_2 dissolved calculated as the product of the CSF

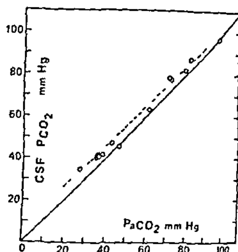


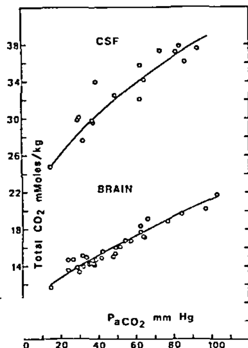
Fig 1 Relation between the measured arterial CO_2 tension and the CO_2 tension of cisternal cerebrospinal fluid in rats anesthetized with nitrous oxide and given 100 mg/kg of acetazolamide i p The oblique uninterrupted line is the line of equality and the interrupted line the arterial plasma/CSF pCO_2 relation in cats not given acetazolamide (Ponten and Siesjo 1966)

weight) and the bicarbonate contained in the extracellular fluid of the tissue (assumed to occupy 12 % of the brain weight) The water content of the present brain material (78 5 %) did not differ from that of uninjected rats For details of calculation see Kjallquist Nard[illegible] and Siesjo (1969 a)

Results

In 31 of the experiments the tissue CO_2 content was measured, and in 25 out of these CSF was sampled for analyses of pCO_2 or of the total CO_2 content There were thus 6 expts in which no CSF was sampled The results obtained in those experiments did not differ significantly from those in which CSF was simultaneously sampled A few animals were excluded from the material Thus if the CO_2 tension measured in arterial blood before and after the sampling of CSF differed by more than 10 % the experiment was discarded Originally CSF was sampled in a larger number of animals for measurements of the total CO_2 content but only those 15 were accepted in which the CSF sample weighed 50 mg or more Finally one experiment was discarded in which the measurement of the CSF CO tension gave a grossly aberrant negative CSF/plasma difference

Fig 1 shows the relation between the measured CO_2 tensions in arterial blood and in cisternal CSF the oblique line being the line of equality and the interrupted oblique line the CSF (and tissue) CO_2 tensions calculated from the results obtained by Ponten and Siesjo (1966) on cats The results confirm the arterial/CSF pCO relations previously reported for normocapnic rats treated with acetazolamide (Brzezinski *et al* 1967 and they show that the CSF CO_2 tension is related to the measured (apparent arterial CO_2 tension in hyper and hypocapnia in a similar way as in animals not treated with acetazolamide On the basis of the results the



[illegible] were drawn by inspection.

CSF (and mean tissue) CO_2 tensions in all subsequent experiments were calculated by adding 5 mm Hg to the measured arterial CO_2 tensions

Fig 2 shows the relation between the measured arterial CO_2 tension and the total CO_2 content of cisternal CSF as well as of the supratentorial parts of the brains In the figure, the values obtained should be compared to lines drawn as best fits to values measured under similar conditions in rats not treated with acetazolamide (Kjällquist, Nardini and Siesjö 1969 a, Fig 1) The comparison shows that the slope of the tissue TCO_2/pCO_2 line, *i e* the change of the tissue CO_2 content with the arterial CO_2 tension, was considerably flatter with acetazolamide than without (*cf* Fig 3), but also that the acetazolamide values were generally much higher than in the uninjected series (see Discussion and Kjällquist Nardini and Siesjö 1969 b) The corresponding TCO_2/pCO_2 line for the cisternal CSF was also flatter than that obtained on rats which were not given acetazolamide (see Fig 2)

Lines of best fit were drawn through the values for the CSF and the tissue CO_2 contents, and the intracellular bicarbonate concentrations were calculated as described in a previous paper (Kjallquist, Nardini and Siesjo 1969 a) The result of this calculation is shown in Fig 3, which relates the CSF and the calculated intracellular bicarbonate concentrations to the tissue CO_2 tension The figure shows the marked difference in the calculated tissue bicarbonate concentrations between the present acetazolamide material (uninterrupted line) and the previous uninjected material (interrupted line) When the bicarbonate and the pCO_2 values were used

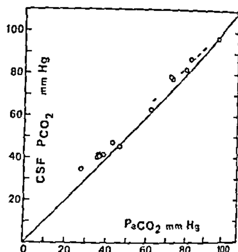


Fig 1 Relation between the measured arterial CO_2 tension and the CO_2 tension of cisternal cerebrospinal fluid in rats anesthetized with nitrous oxide and given 100 mg/kg of acetazolamide i p The oblique uninterrupted line is the line of equality and the interrupted line the arterial plasma/CSF pCO_2 relation in cats not given acetazolamide (Ponten and Siesjo 1966)

CO_2 tension and the sol concentration was derive kg/mm Hg (Siesjo 1962 tracting the bicarbonate weight) and the bicarbonate contained in the extracellular fluid of the tissue (assumed to occupy 12 % of the brain weight) The water content of the present brain material (78 5 %) did not differ from that of uninjected rats For details of calculation see Kjallquist Nardin and Siesjo (1969 a)

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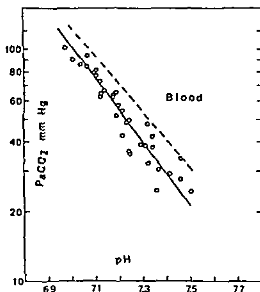


Fig 5 *In vivo* CO_2 buffer curve of arterial blood in animals given acetazolamide as compared to the buffer capacity of uninjected rats (interrupted line) The calculated buffer capacities were rather similar for the acetazolamide groups (β= 1 4 and for the uninjected rats (β=1 3)

In Fig 5 the pH/pCO values for arterial plasma from the present acetazolamide rats have been plotted and compared to the corresponding values for uninjected rats (interrupted line) In both groups the calculated buffer capacity amounted to 1 3—1 4 but the line for the acetazolamide animals was shifted about 5 base excess units to the left of the normal line

Discussion

The present results have shown that the *in vivo* CO_2 buffer capacity of brain tissue calculated as $\beta = \Delta \log pCO_2/\Delta pH$ is markedly lower in animals treated with acetazolamide than in uninjected animals exposed to the same CO_2 tensions for the same time periods (40—60 min) The difference in buffer capacity is so large that the conclusion should be unaffected by any errors in the assumptions regarding the mean CO tension and in the size of the extracellular fluid of the tissue Thus the derivation of a mean tissue CO_2 tension after Diamox is based on an empirical relation between the pCO_2 in arterial plasma and in cisternal CSF (Brzezinski Kjallquist and Siesjo 1967 and Fig 1) and the use of a 12 % extracellular space for the calculation of intracellular bicarbonate concentrations does not take into account any possible variation in the size of the total extracellular volume due to the effect of Diamox upon the CSF production (see *e g* Davson 1967) However even if the calculated mean CO tension is in error by several mm Hg or if the size of the extracellular space is considerably lower than the assumed one the conclusion that acetazolamide lowers the intracellular buffer capacity seems to be valid

There was no indication that the CSF buffer capacity was significantly decreased by acetazolamide Thus the small difference observed may be due to the fac

the rate of change in the CSF bicarbonate concentration after a change in the CO_2 tension is slowed down concomittantly to the lowering of the CSF production by acetazolamide (see Maren 1967). The effect of acetazolamide on the regulation of the CSF pH in response to variations in pCO_2 must evidently be studied with longer equilibration time. It should also be remarked that acetazolamide apparently did not alter the *in vivo* CO_2 buffer capacity of whole blood during the times studied.

The lowering of the CO_2 buffer capacity of the cells by acetazolamide can be explained if it is assumed that the drug interferes with an active transport regulation of the intracellular pH' of carbonic anhydrase containing cells in the brain. Thus there are reasons to believe both that there exists transport mechanisms which extrude hydrogen ions from cells, and that the efficiency of such mechanisms may partly depend on the presence of the enzyme carbonic anhydrase. Let us first discuss the evidence for the presence of a cellular transport of hydrogen ions. The net passive flux of hydrogen ions between the extra- and intracellular spaces of any cell should be proportional to the electrochemical potential difference for hydrogen ions

$$\Delta\mu_{H^+} = RT \ \ln \frac{(H^+)_i}{(H^+)_o} + F \ (\Psi_i - \Psi_o)$$

where $\Delta\mu_H$ is the electrochemical potential difference and Ψ the electrical potential difference across the plasma membrane, R the gas constant, F the Faraday and T the temperature, and where i and o denote the intra- and extracellular space respectively. In this equation we neglect any hydrostatic pressure gradients and any osmotic gradients giving a solvent drag effect (see Ussing 1965). If we set the temperature to 37° C and if we regard the potential of the extracellular space as zero we can write

$$\Delta\mu_{H^+} = 61.5 \ (pH_o - pH_i) + \Psi_i$$

Nerve and muscle cells in general have negative intracellular potentials of 60 to 90 mV and most measurements or calculations have given pH values of 6.9–7.2 at extracellular pH values of 7.3–7.4 (see *e.g.* Hill 1955, Caldwell 1956, but also conflicting results by Kostyuk and Sorokina 1960). At an intracellular potential of −75 mV, an intracellular pH of 7.1 and an extracellular pH of 7.4 we can calculate a $\Delta\mu_H$ of −57 mV. This means that there is a net passive force tending to drive hydrogen ions into the cells. If unopposed, this force will tend to equilibrate the hydrogen ion concentration in the cell with the extracellular hydrogen ions. At equilibrium, when $\Delta\mu_H$ is zero, the intracellular pH would be about 6.1. The fact that the intracellular pH, is around 7 indicates that there is a transport of hydrogen ions out from the cells balancing the passive influx along the electrochemical gradient.

If we accept an active transport regulation of intracellular pH we must inquire about the mechanisms involved. Although the molecular processes remain enigmatic there are indications from renal physiology that the triggering mechanism may be the CO_2 tension, and that carbonic anhydrase is involved (Brazeau and Gilman

1955 Relman Etsten and Schwartz 1953, Clapp Watson and Berliner 1963, Pitts 1966) Thus although the hydrogen ion transport by the kidney cells appears to be a transcellular event due to a functional assymmetry of the tubulus membranes, the rate of transport may be set by the change in intracellular pH of the cells (Pitts 1966) On the basis of available evidence we may then envisage a mechanism where metabolic energy is used to split water into H and OH (Mitchell 1962, 1966, Robertson 1968), and where hydrogen ions are delivered to the exterior (*cf* Siesjö and Kjallquist 1969) If the rate limiting step in the transport process is the removal of the hydroxyl ions by means of combination with CO_2 in the membranes, or in the cytoplasm the rate of H^+ transport may vary directly with the CO_2 tension, and may be inhibited by carbonic anhydrase inhibitors Thus, if we momentarily increase the CO_2 tension of the system the decrease in pH_i will initially only be limited by the intracellular buffers present However, the increased CO_2 tension will increase the number of hydrogen ions available to a transport system which could increase the active H^+ outflux until the new rate of transport again balances the passive influx. At this time, the pH_i' will have been regulated to a value intermediate between the original pH_i' and that obtained in the beginning of the hypercapnic period

A H transport system as discussed could explain why the *in vivo* CO_2 buffer capacity of brain tissue is higher with nitrous oxide anesthesia (Kjallquist, Nardini and Siesjo 1969 a) than in deep phenobarbital anesthesia (Ponten 1966), and than in nitrous oxide anesthesia with simultaneous carbonic anhydrase inhibition (present results) Thus in phenobarbital anesthesia the metabolism may be sufficiently depressed to lower an active extrusion of H and in carbonic anhydrase inhibition the delivery of hydrogen ions to an transport mechanism may be diminished If this is the actual mechanism involved, we must assume that cells which contain no carbonic anhydrase, and which thus must rely on the uncatalyzed reaction for CO hydration, either have a lower permeability to H , and thus a lower rate of passive influx of protons or that such cells have a lower pH_i' than carbonic anhydrase containing cells In the brain the latter are formed by plexus chorioideus cells and by glial cells (Giacobini 1962 see also Maren 1967)

Although it is tempting to explain the low CO buffer capacity of brain tissue under carbonic anhydrase inhibition as an interference with an active H transport by acetazolamide, this explanation obviously demands that the intracellular bicarbonate concentration decreases when the drug is given In fact the present results have confirmed the previous finding that the intracellular bicarbonate concentration is increased after acetazolamide an increase which cannot be explained by the small differences in the lactate concentrations at the actual CO tensions (Kjallquist Nardini and Siesjo 1969 b) The easiest way to explain both the effect on the buffer capacity and the effect on the intracellular bicarbonate concentration is to assume that acetazolamide leads to a H_2CO_3 acidosis in the tissue (Severinghaus and Cotev 1968), and to assume that the increased H_2CO_3 concentration is responsible for the increased bicarbonate concentration while the decreased buffer capacity is due to an interference with an active H transport However the presence of a H_2CO_3

acidosis requires that the metabolically produced carbon dioxide is in the form of H_2CO_3, and since this is a matter of controversy (see Discussion by Kjallquist, Nardini and Siesjo 1969 b), the final interpretation of the present results must be left until more information is available on this topic

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The Catechol Amine Content of the Bull Retractor Penis Muscle

By

Erik Klinge

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Abstract

Klinge, E. *The catechol amine content of the bull retractor penis muscle.* Acta physiol. scand. 1970. 78. 103—109.

The noradrenaline, adrenaline and dopamine content of the bull retractor penis muscle was determined. Dowex 50 X4 ion exchange columns were used for the purification procedure and for the separation of dopamine from the other catechol amines. The muscle contains considerable amounts of noradrenaline but it probably is devoid of adrenaline. The dopamine content is about 10 % of that of noradrenaline. It seems possible that the impulses maintaining the almost continuous contraction of the bull retractor penis muscle are conducted along sympathetic nerves.

A sketch of the dog retractor penis was given by Eckhard in 1863. One of the first detailed investigations into the physiology of the retractor penis muscle of several mammalian species including the bull was probably that made by Sertoli in 1883. After him most physiologists examining this muscle have been dealing with the dog. The dog retractor is powerfully contracted by stimulation of the lumbar sympathetic chain or the pudic nerve (Langley and Anderson 1895) as well as by i.v. injection of adrenaline (Elliott 1905). These effects are prevented by ergot (Dale 1906). Brucke (1910) registered continuous waves of action potentials in the contracted dog retractor that were strengthened on sympathetic stimulation (Brucke and Oinuma 1910). These observations prompted several extensive studies of the nervous control of the dog retractor penis (*e.g.*, Bottazzi 1916, Edmunds 1920, Luduena and Grigas 1966). But so far at least the peripheral nervous mechanism of relaxation remains poorly understood, although it was demonstrated by Eckhard as early as in 1863 that the muscle is relaxed on stimulation of certain sacral autonomic nerves which he called nervi erigentes (also called pelvic nerves or sacral parasympathetic nerves).

The present work was undertaken to study the possible role of the sympathetic nervous system in maintaining the almost continuous contraction of the bull re-

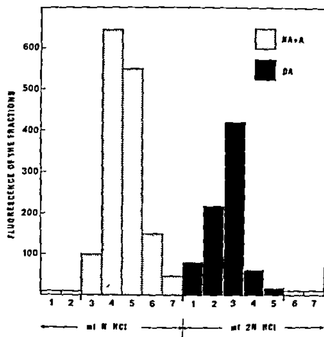


Fig 1 Elution pattern of adrenaline (A), noradrenaline (NA) and dopamine (DA) from Dowex 50-X4 columns, 200 to 400 mesh size 12 6 mm² × 52 mm, H form. Elutriant N HCl and 2N HCl 0 25 ml per min The relative fluorescences of the 1 ml fractions are the mean values of 10 determinations performed according to Haggendal (1962) The same results were obtained after oxidation of the catechol amines

tractor penis muscle Strips of this muscle suspended in Tyrode solution are vigorously contracted by low concentrations of adrenaline and noradrenaline (Klinge 1967 1969c) and of bradykinin (Klinge 1969d)

Material and methods

General principles The technique used for the quantitative determination of noradrenaline (NA) and adrenaline (A) is based on the principles described by Bertler Carlsson and Rosengren (1958) and by Vendsalu (1960) The dopamine (DA) content was estimated according to Carlsson and Waldeck (1958) A more detailed description of all the steps is given elsewhere (Klinge 1969e)

Preparation of extracts Fresh samples of the retractor muscle obtained from Ayrshire bulls weighing 150—250 kg were homogenized under cooling in 0 4 N perchloric acid with an 'Ultra Turrax' homogenizer It was checked that the results were the same as when the tissues were ground in a mortar The homogenates were handled as described by Klinge (1969e)

Column procedure After removing of the potassium perchlorate by filtration the clear filtrates were passed through ion exchange columns details of which are stated in the text of Fig 1 Before elution the columns were rinsed with 10 ml of 0 1 % ethylene diamine tetra acetic acid (EDTA) 5 ml of 0 1 M phosphate buffer pH 6 5 and 10 ml of deionized and distilled water The elution was performed as illustrated in Fig 1

Spectrophotofluorometry The pH of the first eluate of 5 ml was adjusted to 6 4 with 5 N potassium carbonate An automatic titrator (Radiometer TTT 1b) was used An aliquot of 2 0 ml was taken and 0 3 ml of 0 1 M phosphate buffer pH 6 5 and 0 65 ml of water were added The oxidation was performed with 0 05 ml of 0 25 % potassium ferricyanide 5 min later 1 ml of a newly prepared mixture containing 5 N sodium hydroxide ethylene diamine (EDA) and 2 % ascorbic acid (8 8 0 2 1 0 v/v) was added (Euler and Lishajko 1961) After another 5 min the fluorescences of the lutines of NA and A were measured at 400/510 nm and at 440/540 nm respectively (uncorrected instrumental values) A standard Aminco-Bowman spectrophotofluorometer was employed

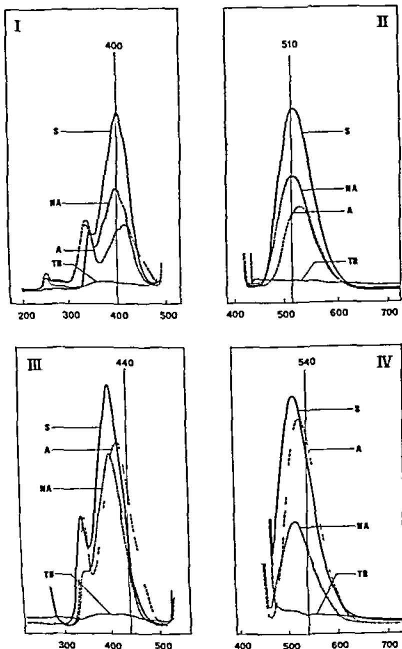


Fig 2 Activation and fluorescence spectra of the first eluate of a bull retractor penis sample (S) and of noradrenaline (NA) adrenaline (A) and the tissue blank TB after oxidation and rearrangement in alkali

I Activation spectrum Fluorescent wave length 510 nm.
II Fluorescence spectrum Activating wave length 400 nm
III Activation spectrum Fluorescent wave length 540 nm
IV Fluorescence spectrum. Activating wave length 440 nm

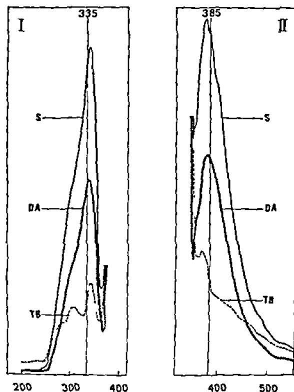


Fig 3 Activation and fluorescence spectra of the second eluate of a bull retractor penis sample (S), of dopamine (DA) and of the tissue blank (TB) after oxidation and rearrangement to the more fluorescent dihydroxyindole

I Activation spectrum Fluorescent wave length 385 nm
II Fluorescence spectrum Activating wave length 335 nm

For the assay of DA the pH of the second eluate of 5 ml was adjusted to 6 5 with 5 N potassium carbonate An aliquot of 2 0 ml was put into a silica test tube and the procedure was continued as described by Carlsson and Waldeck (1958) The fluorescence was read at ~/385 nm DOPA (3 4 dihydroxy phenylalanine) did not interfere with the estimation of DA With the present technique about 50 % of the DOPA put on the column was washed out before elution was begun and the rest was found in the first 4 ml of the eluate obtained with N HCl

Standards and recoveries An internal standard was always r... The internal standards th... the reagent blanks were ... salu (1960) With the ret... of DA 80 ± 13 % If the ... 11—16 % higher All res... ...ed for the respective recoveries

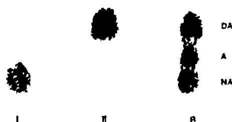
Activation and fluorescence spectra Both spectra of the eluate containing the NA and A of the muscle were exactly identical with those of the NA standard (Fig 2) If they contained the same amount of NA their spectra were indistinguishable when drawn on the same paper The spectral maxima of the DA eluate corresponded to those of the DA standard (Fig 3)

Paper chromatography The chromatography was carried out according to Bertler *et al* (1958) and the chromatograms were stained according to Ellman (1958) The first eluate gave only one spot corresponding to NA (Fig 4) In nine runs no traces of A were observed though the amount of NA in the eluate was 4—5 μg The second eluate gave only one spot corresponding to DA

Results

The results are illustrated in Table I Considerable amounts of NA were found in all parts of the muscle It probably did not contain any A It must, however, be kept in mind that with the present technique minute amounts of A can have been masked

Fig 4 Chromatogram of the retractor penis muscle eluates Paper Whatman no 1, solvent n butanol HCl, temperature +24° C, ascending time 12 hrs staining with 0 1 % potassium ferricyanide in 5 % aqueous ethylene diamine I From the first eluate II From the second eluate R References NA Noradrenaline A Adrenaline DA Dopamine The references correspond to 0 5 μg of each catechol amine base



by large amounts of NA This is due to the small difference between the spectra of adrenolutine and noradrenolutine The DA content was about one tenth of that of NA Both the NA and the DA content decreased towards the distal end of the muscle

Discussion

Some unusual characteristics are associated with the function of the retractor penis muscle The task of the muscle is to keep the relaxed penis under the skin Accordingly, it is almost continuously contracted, being probably relaxed only at penile erection The observations mentioned in the introduction of this paper suggest that the sympathetic nervous system is involved in maintaining the contracted state of the dog retractor penis In this animal the adrenergic fibres reach the muscle mainly along the pudic nerve and to a minor extent along the hypogastric nerve directly via the pelvic (hypogastric) plexus (Langley and Anderson 1895)

In the present study large amounts of NA were found in the bull retractor penis Its NA content probably is exceeded only by that of the vas deferens the seminal vesicle and the prostate provided that the adrenal glands and certain sympathetic nerve trunks are not taken into account (Euler 1948 Sjostrand 1965 Klinge 1969e) On the other hand the DA content of the retractor muscle is much lower than that of several bovine tissues in which non neuronal DA containing cells have been detected (*e g* Bertler *et al* 1959 Schumann 1959) It remains to be solved whether DA has any function in the retractor other than to serve as precursor of NA It is commonly accepted that the quantity of NA in a peripheral organ reflects the density

TABLE I Catechol amine content of different parts of the retractor penis muscle of eight bulls expressed in μg/g of tissue (mean ± S D)

Part of muscle	Noradrenaline	Adrenaline	Dopamine
Proximal	3 8 ± 0 53	<0 02	0 37 ± 0 07
Middle	3 7 ± 0 69	<0 02	0 34 ± 0 07
Distal	1 9 ± 0 21	<0 01	0 20 ± 0 03

of adrenergic nerve fibres, the abundance of which in the bull retractor penis has recently been confirmed by fluorescence microscopy (Klinge, Pohto and Solatunturi 1969). The presence of alpha adrenergic receptors in the muscle has been established (Klinge 1967, 1969c). These data indicate that also in the bull the sympathetic nerves may play an important role in maintaining the almost continuous contraction of the retractor penis muscle. The activity of the enzymes known to inactivate the adrenergic transmitter substance is considered in another paper (Klinge 1969b). The possible mode of action of the autonomic nervous system in the regulation of the tonus of the muscle is also discussed in more detail in separate reports (Klinge 1969a, 1969c).

Only minute amounts of NA have been observed in the corpora cavernosa penis and the corpus cavernosum urethrae of the bull (Penttilä and Vartiainen 1964, Penttilä 1966). The obvious absence of adrenergic nerve fibres in these tissues has been confirmed by histochemistry (Klinge and Penttilä 1969). Striking differences appear to exist in the intensity of the adrenergic innervation in the different external generative organs of the bull. The functional implications of these variations are discussed in another connection (Klinge 1969c).

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Acta physiol. scand. 1970. 78. 110—116

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Adrenergic Innervation and Structure of the Bull Retractor Penis Muscle

By

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Abstract

KLINGE, E, P POHTO and E SOLATUNTURI *Adrenergic innervation and structure of the bull retractor penis muscle* Acta physiol scand 1970 78 110—116

The structure of the retractor penis muscle of the bull was examined by staining with hematoxylin and eosin The autonomic innervation of the muscle was investigated by applying formaldehyde induced catechol
the original thiocholine metho
smooth muscle fibres only A
was also found in the nerves T
the nerves showed some corres
discussed

The physiological role of the paired retractor penis muscle of several mammalian species including the ox has been clearly defined by Kolliker in 1852 The almost continuous contraction of this muscle retains the bovine penis in a curved position under the skin In bulls of the Ayrshire breed weighing about 200 kg one retractor muscle has a weight of 22—30 g and in its usual contracted state a length of 55—65 cm At erection the muscle relaxes and the penis becomes straight and filled with blood After complete relaxation the length of the muscle is increased by 50—60 % In addition to this uncommon mode of function of the retractor penis muscle there are certain earlier observations which are of particular interest

Langley and Anderson (1895 and De Zilwa (1901) reported that varying amounts of striated fibres are mixed in the proximal end of the dog retractor penis though the main part of the muscle is composed of smooth fibres Their observation was confirmed by Fisher (1917 The structure of the bull retractor is still disputed According to Retterer (1915) and Ackerknecht (1913) it is a striated muscle whereas Sisson (1959) holds the opinion that it is a smooth one

The existence of an abundant nervous network in the rat retractor was reported by Fletcher in *1898 The investigations of Langley and Anderson* (1895), Elliott (1905) and Dale (1906) revealed that the contractile impulses for the dog retr[illegible]

are conducted along sympathetic nerve fibres. Recently large amounts of noradrenaline (NA) were detected in the bull retractor (Klinge 1969a). The purpose of the present work has been to confirm the structure of the muscle fibres of the bull retractor penis and to study its autonomic innervation by histochemical methods.

Methods

Formaldehyde induced catechol amine fluorescence. The principles outlined by Eranko (1967a) were followed. Small pieces of tissue were rapidly frozen in isopentane precooled with solid CO_2 in acetone. They were dried in vacuo (10^{-6} mm Hg at the end of the procedure) at —35° to —40° C for 2—4 days. The best results were obtained after immediate exposure to formaldehyde vapour at 60° C for 1—2 hrs at a relative humidity of 70 %. The tissues were embedded in paraffin wax under vacuum after immersion in xylene. Sections of 10—20 μm were prepared. The specimens were examined with a Carl Zeiss Mf microscope equipped with a mercury lamp (Osram HBO 200), UV filter, heat absorption filters, excitation filter (Schott BG 12, 4 mm), bright field or oil immersion dark field condenser and barrier filter (Schott OG 4, 3 mm).

Cholinesterases. The localization of the cholinesterase activity was performed according to the copper ferricyanide method of Karnovsky and Roots (1964) which is a modification of the original thiocholine method of Koelle and Friedenwald (1949). The tissues were fixed for 24 hrs in cold 10 % formalin containing 1 % $CaCl_2$. Subsequently they were blotted dry, dropped in O.C.T. (LAB-TEK, Westmont, Illinois), and quick frozen on a freezing stage cooled with CO_2. Sections of 10—20 μm were cut at −15° C. Before starting of the prein-
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(1,5-bis/N-allyl-N,N-dimethyl-4-ammoniumphenyl/pentan-3-one dibromide, Wellcome Research Laboratories). For selective inhibition of the nsChE IsoOMPA (teramonoisopropyl pyrophosphortetramide, Koch Light Laboratories Ltd) was identically used. The incubation times at 37° C were 30 min and from 1 to 18 hrs respectively.

For photographing Agepe FF (Agfa Gevaert) and Agfa CK or CT 18 films were used.

Results

Hematoxylin and eosin. No striated fibres were observed in any part of the muscle. The shape and arrangement of nuclei corresponded to that of smooth muscle (Fig. 1 and 6). Some nuclei seemed pleated along their longitudinal axes as indication of the contracted state of the muscle. There were considerable amounts of connective tissue between the bundles of muscle fibres (Fig. 6). The thick-walled arteries supplying the muscle ran along the connective tissue but were not as numerous as the concomitant nerve trunks that stained with the other technique.

Formaldehyde induced catechol amine fluorescence. The existence of a rich adrenergic innervation consisting of parallel thin spiral shaped nerve fibres is illustrated in Fig. 4 and 5. The fluorescence intensity increased towards the end of the fibres. In transverse sections the fluorescent nerve fibres gave the impression of a star map (Fig. 7). No non-neuronal monoamine cells could be detected.

Cholinesterases. If the incubation time was shorter than 2 hrs AChE activity was observed only in the large nerve trunks running along the connective tissue. The nerve trunk seen in Fig. 2 exhibited the same spiral configuration as the blood vessels. After an incubation of 8 hrs AChE was stained also in the minor nerve branches or single axons running between the muscle fibres (Fig. 9). Nothing like the endplate at spinal nerve endings or other special terminal structures was observed with the



Fig 1 Bull retractor penis muscle Hematoxylin and eosin The elongated nuclei show a configuration and arrangement typical of smooth muscle Longitudinal section from the proximal end 15 µm, × 200

Fig 2 Acetylcholinesterase A nerve trunk with the characteristic spiral shape corresponding to the contracted state of the muscle Incubation time 8 hrs Longitudinal section 20 µm × 250

Fig 3 Non specific cholinesterase Some thin nervous structures (arrows) are more intensely stained than the surrounding muscular tissue Incubation time 8 hrs Longitudinal section 15 µm × 110

present technique The nsChE activity was always almost absent in the large nerve trunks and in the blood vessels but there was moderate activity in the sarcoplasma and in the cellular membranes (Fig 8) Following an incubation of 8 hrs a distinct nsChE activity was observed in or around some thin bundles of nerve fibres or single axons (Fig 3 and 8

In adjacent sections there was certain correspondence between the NA and AChE positive fibres The latter however seemed slightly outnumbered by the former In general there seemed to be approximately an inverse correlation between the distribution of NA and AChE in the longitudinal direction of the bundles of nerve fibres Nothing that would resemble autonomic ganglion cells could be observed with any of the staining principles employed

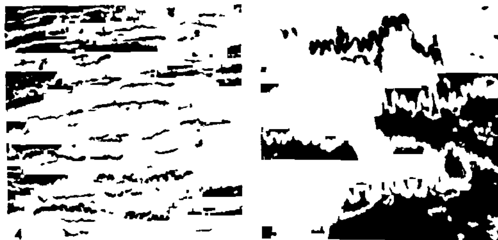


Fig 4 Formaldehyde induced fluorescence A dense adrenergic innervation consisting of spiral shaped parallel nerve fibres is seen Longitudinal section 20 μm × 120

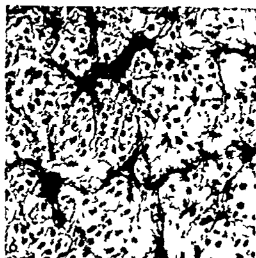
Fig 5 Same as Fig 4 The spiral shape of the nerve fibres is better illustrated 15 μm × 400

Discussion

The lack of striated fibres in the proximal end of the bull retractor penis muscle is consistent with the fact that in this animal the muscle is wholly plain and constitutes a separate entity that takes its origin on the ventral surface of the bulbocavernosus muscle Here it lies in a continuation of the anal suspensory ligament Along this ligamental sheet only blood vessels and nerves reach its origin The characteristically spiral shape of the arteries and nerves reflects the capacity of the muscle to become considerably lengthened

The existence of numerous NA positive nerve fibres is in accordance with the large amount of NA found in the muscle It is likely that the excitatory nerve impulses causing the almost continuous contraction of the muscle are mediated via these fibres This assumption is supported by the preliminary observation that the isolated muscle is powerfully contracted by NA or adrenaline (Klinge 1967) It remains to be solved whether the adrenergic neurons emanate from some of the deep pelvic ganglia described by Muller (1835) and in more detail by Owman and Sjostrand (1966), or from para or prevertebral ganglia The obvious absence of autonomic ganglia within the muscle is further strengthened by the finding that isolated muscle strips are poorly affected by nicotine (Klinge 1969c)

In the present study the same sections were not examined for both NA and AChE A comparison of adjacent sections indicated some but not full correspondence between the location of the NA fluorescence and the AChE activity In the case of correspondence it is not possible according to the observations of Richardson (1964) Jacobowitz and Koelle (1965) and Eranko (1967b) to evaluate solely by means of light microscopy whether the NA and the AChE are located in the same or in concomitant axons Therefore an electron microscopic study would be interesting



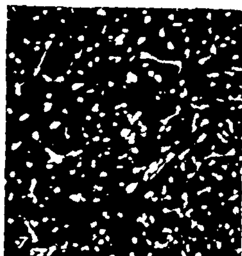






Fig 6 Hematoxylin and eosin Transverse section The distribution of nuclei corresponds to that of smooth muscle Connective tissue appears as irregularly stained dark spots between the bundles of muscle fibres 20 μm ×300

Fig 7 Formaldehyde induced fluorescence Transverse section The evenly distributed adrenergic nerve fibres give the impression of a star map 20 μm ×150

Fig 8 Non-specific cholinesterase Transverse section The large nerve trunks (arrows) are almost unstained but several thinner nervous structures are well stained Incubation time 8 hrs 15μm ×210

Fig 9 Acetylcholinesterase Transverse section The large nerve trunks show the most intense staining but many thinner nerve branches are also well stained Incubation time 8 hrs. 15 μm ×150

The tentative assumption can be made that in a mammalian tissue like the retractor penis muscle AChE reflects the associated presence of acetylcholine (Koelle 1963) It was observed that the AChE activity was lowest at the end of the nerve fibres and increased in the central direction Nevertheless the AChE activity might

be similar along the whole postganglionic fibre and a false impression could arise because of several joined fibres running concomitantly. The correctness of the observation would not support the conception that acetylcholine is liberated from the nerve terminals as a transmitter of the impulses. The feeble effect of acetylcholine upon the isolated muscle (Klinge 1967, 1969c) is also incompatible with the presence of any kind of acetylcholine receptors in the muscle cells. However, the possible role of acetylcholine as well as the validity of the hypothesis of Burn and Rand (1959) will be considered in a further paper (Klinge 1969b).

The significance of the moderate but distinct nsChE activity detected in or around some nerve fibres can not be explained without additional studies. It seems unlikely that this staining was effected by AChE since the large nerve trunks did not stain. The major portion of the nsChE activity in question probably is confined to glial elements, i.e., to Schwann cells, and a minor part might be found in the axons. This localization corresponds to that observed by electron microscopy in nerves in the rat uterus (Teräväinen 1969). The morphology of the nsChE positive sensory corpuscles in the skin of the human penis (Hurley 1958) is quite different from that of the structures stained for this enzyme in the present study. But so far it is not excluded that a homologue of acetylcholine is involved in the function of the efferent nerves in the bull retractor. This speculation is rendered more interesting by the fact that the ox spleen is, to the authors' knowledge, the only mammalian tissue from which the isolation of an ester of choline other than acetylcholine has been successful (Banister, Whittaker and Wijesundera 1953, Whittaker 1963). The ester in question was identified as propionylcholine, but its physiological role is as yet unknown.

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Acta physiol. scand 1970 78 117—122
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On the Mechanism of Depletion of Noradrenaline Stores by Iso-Monomethylnicotinium Bromide

By

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Abstract

HEDQVIST, P *On the mechanism of depletion of noradrenaline stores by iso monomethylnicotinium bromide* Acta physiol scand 1970 *78* 117—122

The quaternary nicotine analogue *iso* monomethylnicotinium bromide (IMN) inhibits the uptake of noradrenaline (NA) in the perfused guinea pig heart without causing any change in the distribution between the particulate and soluble fractions of the NA taken up In the isolated perfused cat spleen IMN increases the resting outflow of NA and the NA overflow response to nerve stimulation while that to tyramine (TA) is reduced IMN does not affect release or uptake of NA in isolated splenic nerve granules, or the formation of NA from tyrosine in this system

It is suggested that IMN causes depletion of NA stores both by releasing NA probably from extragranular sites and by inhibiting reuptake of the NA released

The quaternary nicotine analogue IMN has recently been shown to cause a marked depletion of the NA content of a variety of organs (Euler *et al* 1969) In this respect IMN resembles nicotine which has been reported to reduce the NA content of several organs (Westfall 1965) On the other hand IMN has previously been found almost to lack the blood pressure increasing effect of nicotine on the spinal cat (Larsson and Haag 1943 Barlow and Dobson 1955, Gillis and Lewis 1956)

In order to obtain more information about the mechanism of the NA depletion the effect of IMN was studied on the uptake of NA in the perfused guinea pig heart, on the release of NA from the isolated perfused cat spleen on the release and uptake of NA in isolated splenic nerve granules and on NA synthesis in splenic nerves

Methods

Uptake of NA in isolated guinea pig heart

Guinea pigs were given IMN (300 mg/kg) intraperitoneally and sacrificed one hour later The heart was removed and perfused by the Langendorff technique with Krebs-Henseleit s solution After infusion of ^{3}H dl NA (2.5×10^{-8} M) for 10 min the heart was washed with

Abbreviations used IMN—*Iso* monomethylnicotinium bromide NA Noradrenaline DA= Dopamine TA=Tyramine

NA free medium for 5 min and then homogenized in 0 4 M perchloric acid and analyzed for radioactivity

In some experiments the perfused heart was homogenized in icecold isotonic phosphate buffer pH 7 5 and centrifuged at $9000 \times g$ for 10 min The low speed supernatant was then centrifuged at $100\,000 \times g$ for 45 min providing two more fractions, particulate (high speed sediment) and soluble (high speed supernatant) The different fractions were extracted with perchloric acid and analyzed for radioactivity

Release of NA in isolated spleen

Spleens from 5 cats were isolated and perfused at a constant rate of 7 5 ml/min with a modified Krebs Henseleit s solution as previously described (Hedqvist and Stjarne 1969) The spleen was loaded with ^{3}H dl NO (35 μC, spec act 5 C/mmole) and about 1 hr later exposed to supramaximal nerve stimulation (10/sec for 30 sec) or to i a injection of TA (20 μg) with or without IMN (200 μg/ml) in the perfusion medium The NA outflow from the spleen was monitored by following the output of radioactivity in each 10 ml fraction of the venous effluent while the quantitative determination of the NA outflow was based on fluorimetric assay of NA in pooled effluent fractions

Release and uptake of NA in isolated nerve granules

ld 0 13 M potassium phosphate buffer and 10 min at $9000 \times g$ 8 ml fractions of the issue were incubated for 60 min at 20° C M After the incubation the suspension was centrifuged for 30 min at $50\,000 \times g$ The sediment and supernatant were extracted with 0 4 M perchloric acid The radioactivity of the extract was determined as described below Total NA was assayed fluorimetrically The NA content of the sediments was compared to that g the incubation period lated as the ratio of the high speed centrifuga

NA synthesis in nerve tissue

Of the low speed supernatant from homogenates of bovine splenic nerves (*cf* above) 8 ml portions corresponding to about 1 g of tissue, were incubated with or without IMN (10^{-4} M) in stoppered glass centrifuge tubes at 20° C for 60 min in the presence of 8 μC of tritium labelled tyrosine (New England Nuclear Corp l tyrosine 3 5 H^3 specific activity 4 C/mmole) Adenosine triphosphate 3 mM and magnesium sulphate 3 mM were also added The IMN tubes were preincubated with the drug for 30 min at 0° C

After the incubation period the particle suspension was sedimented and extracted with ice cold perchloric acid. After purification on aluminium oxide the eluate was fractionated by cation exchange column chromatography (Stjarne and Lishajko 1967) The positions of added carrier NA and dopamine (DA) were determined by reading the spontaneous fluorescence in an Aminco-Bowman spectrophotofluorimeter Radioactivity was measured by counting aliquots of the fractions in a Packard tri-carb spectrometer (*cf* below)

Analysis of radioactivity

Radioactivity was determined by counting aliquots in a Packard Liquid Scintillat on Spectrometer using a 7 3 toluene absolute ethanol mixture containing 4 g of 2 5 diphenyloxazole and 100 mg of 1-4 bis 2 4 methyl 5 phenyloxazolyl)benzene per liter of toluene Samples were counted for 10 min Quenching was monitored with internal standards

NA assay

The NA content of perfusate samples and tissue extracts was assayed fluorimetrically after purification on aluminium oxide (Euler and Lishajko 1961)

Results

Perfused guinea pig heart

In perfused guinea pig hearts the amount of radioactivity remaining in the hearts 5 min after infusion of 5 μC of ^{3}H-dl NA was $294\,000 \pm 11\,000$ cpm/g (mean ± SEM, n=4) The corresponding figures for radioactivity in hearts from animals

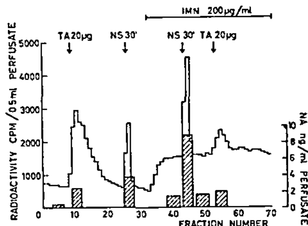


Fig 1 Perfused cat spleen loaded with ^{3}H-dl NA Outflow of radioactivity (drawn line) and of fluorimetrically determined NA (shaded areas) from the spleen resting and in response to nerve stimulation (NS) or TA injection Effect of IMN added to the perfusion medium

pretreated with IMN 300 mg/kg 1 hr before the perfusion was 111,000 ± 11,000 cpm/g mean ± SEM, n=4), suggesting a marked inhibition of NA uptake and/or retention in the hearts from drug treated animals The ratio of particulate to soluble radioactivity in the hearts was 0 67 (range 0 63–0 70, n=2) and 0 62 (range 0 56–0 67, n=2) in control and IMN pretreated animals respectively, and thus did not appear to differ

Perfused cat spleen

In the absence of drug treatment electrical nerve stimulation and i a injection of TA consistently produced an increase in the amounts of both radioactive and fluorimetrically determined NA recovered from the venous effluent of 5 isolated, perfused spleens (*cf* Hedqvist, Oliverio and Stjarne 1968) Addition to the perfusion medium of IMN in a dose of 200 µg/ml markedly increased the spontaneous outflow of radioactivity and of endogenous NA In the presence of IMN the overflow response to TA was found to be reduced, while that to nerve stimulation was increased (Fig 1)

TABLE I Bovine splenic nerve granules incubated in 0 13 M phosphate buffer pH 7 5 for 60 min at 20° C in the presence of IMN 10^{-5} — 10^{-3} M and of ^{3}H-dl NA 5 x 10[illegible] M Remaining NA in sediment (NA_{SED}) after incubation expressed as per cent of original amount Incorporation of ^{3}H-dl NA estimated as the ratio of the specific activities in the sediment and in the supernatant (SA_{SED}/SA_{SU}) Mean values of 2 experiments

IMN molar conc	—	10^{-5}	3×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}
NA_{SED} % of control	50	49	46	50	51	49
SA_{SED}/SA_{SU}	0 28	0 28	0 29	0 27	0 28	0 31

TABLE II. Formation of NA and DA in bovine splenic nerves after incubation with l-tyrosine-³H, (8 μC). Effect of IMN (10^{-4} M).

Number of experiments	Treatment	³H-NA (Mean ± SEM) cpm/g	³H-DA (Mean ± SEM) cpm/g
3	Control	7270 ± 450	8460 ± 660
5	IMN	7560 ± 440	8340 ± 660

Bovine splenic nerve granules

Addition of IMN (10^{-4} M – 10^{-3} M) to isolated bovine splenic nerve granules did not affect the spontaneous release of NA from the granules during incubation in isotonic phosphate buffer. Incorporation of labelled NA into the granules was not affected, indicating undisturbed reuptake of endogenous NA (*cf.* Euler and Lishajko 1967) (Table I).

In bovine splenic nerves IMN (10^{-4} M) did not affect the rate of formation of either NA or DA from tyrosine (*Table II*).

Discussion

In the experiments with perfused guinea-pig hearts the amount of radioactivity recovered from the hearts after infusion of ³H-dl-NA was markedly reduced by IMN (*cf.* Bhattacharya 1968). Since the uptake of NA in adrenergically innervated organs involves at least two steps: transport into the nerve terminals and subsequent binding in the NA stores (Whitby, Axelrod and Weil-Malherbe 1961, Euler and Lishajko 1963 a, b, Lindmar and Muscholl 1964), the effect of IMN may theoretically be due to impairment of uptake at the level of the axonal membrane and/or to interference with binding at the storage sites. However, the finding that the ratio of particulate to soluble radioactivity taken up by the hearts remained unchanged after pretreatment of the animals with IMN makes it seem likely that IMN exerts its effect by blocking NA uptake at the axonal membrane rather than by inhibition of NA binding to the storage particles.

IMN did not affect the rate of release of NA or the incorporation of radioactive NA (reuptake) in isolated nerve granules. In this respect IMN resembles nicotine (Euler and Lishajko 1965).

IMN did not affect the formation of NA and DA from labelled tyrosine in isolated bovine splenic nerve granules. This seems to rule out the possibility that the depleting effect on the NA stores should be due to inhibition of NA biosynthesis.

In the cat spleen experiments addition of IMN to the perfusion fluid increased the NA overflow response to nerve stimulation, possibly by inhibition of reuptake of the NA released (*cf.* Hedqvist and Stjärne 1969). However, a facilitating effect on the release of NA can not be excluded.

Drugs like cocaine and phenoxybenzamine block the sympathomimetic effect of TA (Tainter and Chang 1927, Hedqvist, Oliverio and Stjarne 1968), indicating that transport of TA to the interior of the axon is a prerequisite for the NA mobilizing effect of TA Diminished NA overflow response to TA after administration of IMN, as seen in the present experiments, supports the concept that IMN interferes with the amine uptake mechanism in the axonal membrane

IMN was found to cause an increased outflow of NA from the resting perfused cat spleen which might be the result of inhibition of recapture of NA spontaneously leaking from the nerves Such an effect appears unlikely since cocaine and phenoxybenzamine, known to be potent blockers of NA uptake (Gillespie 1965) do not change appreciably the resting outflow of NA from the spleen (Hedqvist and Stjarne 1969) Thus in addition to the proposed effect on NA uptake, IMN seems to cause the release of NA stored in the nerves The lack of effect on isolated storage particles makes it likely that IMN liberates NA from extragranular sites possibly involving the axonal membrane

Under the present experimental conditions a high concentration of IMN was needed to cause liberation of NA However, pretreatment with reserpine has been found to strongly potentiate the NA liberating effect of IMN Thus, in the spinal cat, a low dose of IMN, which is normally devoid of pressor effects has recently been found to markedly increase the blood pressure after previous administration of reserpine (Euler and Persson 1969)

To summarize, it appears likely that IMN acts both by liberation of NA from the nerves and by inhibition of reuptake of NA released thus causing a depletion of the NA stores which is not wholly compensated for by resynthesis of NA

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Neurotransmitter Releasing Effects of Two Quaternary Nicotine Analogues

By

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Abstract

Euler, U S v, F Haglid P Hedqvist and I Motelica *Neurotransmitter releasing effects of two quaternary nicotine analogues* Acta physiol scand 1970 *78* 123—131

Two nicotine analogues *iso* monomethylnicotinium bromide (IMN) and 1 methyl 3 (pyrrolidinomethyl) pyridinium bromide (MPP) have been studied with regard to their catechol amine releasing effects in the guinea pig and the rat In doses of 10—30 mg/kg intraperitoneally IMN decreased the noradrenaline (NA) content of the guinea pig heart In larger doses up to 300 mg/kg both substances caused 80—90 per cent NA depletion of the heart but not in the brain Maximal effect was reached in 6—12 hrs In the decentralized sub-

It has been shown previously (Westfall 1965, Westfall *et al* 1967) that nicotine in doses of 0 5—1 mg/kg i p causes a moderate reduction of the noradrenaline (NA) content of a variety of organs in the rat The nicotine analog 3 pyrrolidinomethyl pyridine, *which served as the parent substance for one of the quaternary compounds* used in the present study also caused a fall in the organ content of NA when given in a dose of 5 mg/kg i p

During a study of various nicotine analogues it was observed that the two quaternary compounds *iso* monomethylnicotinium bromide (IMN) and 1 methyl 3 (pyrrolidinomethyl) pyridinum bromide (MPP) on injection in guinea pigs caused a fall in the NA content of some organs

Abbreviations NA=noradrenaline IMN=*iso* monomethylnicotinium bromide MPP=1 methyl 3 (pyrrolidinomethyl) pyridinium bromide, VMA=vanilmandelic acid.

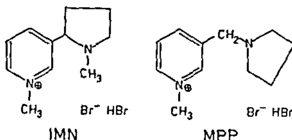


Fig. 1. IMN = *iso-monomethylnicotinium* bromide hydrobromide. MPP = 1-methyl-3-(pyrrolidinomethyl)-pyridinium bromide hydrobromide.

In the present study these effects have been further examined and also the role of nerve activity for the NA releasing action of the compounds and their effect on the excretion of catecholamines in urine. In addition their effects on some isolated organs have been tested.

IMN has previously been studied pharmacologically under the name of nicotine *iso*-methiodide. According to Larson and Haag (1943), Barlow and Dobson (1955) and Gillis and Lewis (1956) it has in mg doses only a feeble, if any, effect on the blood pressure of the spinal cat.

Materials and methods

Preparation of iso-monomethylnicotinium bromide, hydrobromide (IMN) and 1-methyl-3-(pyrrolidinomethyl)-pyridinium bromide hydrobromide (MPP)

0.10 mole of free base (nicotine or 3-pyrrolidinomethyl-pyridine) was added to 0.10 mole hydrogen bromide (5 % aqueous solution). After removal of all water at 50° in vacuum a thick syrup resulted. This was dissolved in absolute methanol (250 ml) and after addition of excess methyl bromide (0.2—0.3 mole) kept in a closed vessel at room temperature for 7 days. Upon removal of solvent, crystalization occurred. The crude product was recrystallized from methanol-methyl ethyl ketone.

IMN m.p. 217—219°

MPP m.p. 195—197° (Fig. 1)

Analysis calculated for $C_{11}H_{18}Br_2N_2$: C 39.08 H 5.36 N 8.28

Found for IMN: C 38.83 H 5.40 N 8.17

Found for MPP: C 38.77 H 5.29 N 8.09

Additional confirmation of structure was obtained from nuclear magnetic resonance spectra. IMN and MPP both (in deuterium oxide with sodium β-trimethylsilyl propionate as internal standard on a Varian A60) exhibited a three proton singlet signal at δ 4.47 ppm from the methyl group on the pyridine nitrogen.

All doses and concentrations refer to the base.

NA content in tissues

For the experiments guinea pigs weighing 250—750 g were used. IMN and MPP were dissolved in a volume of 1.5 ml saline and injected i.p. in doses of 10—300 mg/kg. At different time intervals after the injection the animals were sacrificed by a blow on the head and the ... weighed, homogenized and extracted in 10 % ... bed on alumina and determined fluorimetrically ... alues refer to the free amine (as base) and are ... adrenaline values were in general low in comparison with the NA values and ... included in the results.

Denervation and decentralization experiments

Rats weighing about 200—250 g were anesthetized with 50 mg/kg nembutal and additional ether. The right or left superior cervical ganglion was excised in the denervation experiments and the sympathetic in the neck cut in the decentralization experiments. The NA content of the organs was determined 6 hrs after denervation and 1 1/2—24 hrs after administration of IMN in the animals decentralized 6 days previously.

NA content in rat heart after cold exposure

Male rats weighing about 200 g received IMN (300 mg/kg) intraperitoneally. The animals were exposed to cold (+2° C) or kept in room temperature (+22° C) up to 24 hrs and were then sacrificed and the NA content in the heart determined.

Excretion of NA in urine

Urine was collected during 24 hr periods from single rats in cages coated with plastic material. The urine was acidified in the collection bottle and the catecholamines adsorbed on alumina, eluted and determined fluorimetrically. VMA was determined according to Pisano, Crout and Abraham (1962). IMN was injected in doses of 50, 100 and 300 mg/kg i.p. at the beginning of the 24 hr period.

Isolated organs

The effects of the two compounds were tested on the isolated rabbit jejunum, the guinea pig ileum and vas deferens suspended in Tyrode solution at 38° C and aerated with 5 % CO_2 in O_2. The vas deferens was prepared according to Hukovic (1961) and the hypogastric nerve stimulated for 5 sec at 1 min intervals with supramaximal stimuli of 3 msec duration at a frequency of 20—60 per sec. Tests were also made on the isolated frog rectus abdominis muscle (*Rana temporaria*) in bicarbonate free frog Ringer solution at 22° C, aerated with oxygen.

Results

1. *Effect of IMN on NA content of guinea pig organs*

The NA content of the heart, kidneys and brains in controls showed fairly large variations. In the whole material (n=16) the average value in the heart was 2.16 ±0.16 μg/g (M±S.E.M.). It was noticed that the values were significantly (p=0.05) lower in January—April (1.79±0.17) than in September—December (2.39±0.22). The control values in the kidneys were 0.33±0.026 and in the brain 0.20±0.023 μg NA per g tissue.

Doses of 10 and 30 mg/kg of IMN caused irregular effects on the NA content of the guinea pig organs when determined 3 hrs after injection. In the majority of cases no definite lowering of the NA content was observed but in some cases values were obtained which were considerably lower than those of the controls, both in the heart and kidneys. When measured 6 hrs after injection the effects were consistent, however, both for 10 and 30 mg/kg IMN on the heart (Table I). No effect on the NA content of the organs was noted with MPP in the same doses.

In doses of 50 mg/kg i.p. a considerable reduction of the NA content of the heart was noted with IMN, the lowest values being observed 6 hrs after injection. This effect had largely disappeared 12 hrs after injection. The effect of this dose on the

TABLE I. Noradrenaline content μg/g (M±SEM) in guinea pig heart, kidneys and brain in controls and after injection of 10 and 30 mg/kg IMN intraperitoneally in 1.5 ml saline. Organs removed 6 hrs after injection.

	Heart	Kidney	Brain
Control	2.16±0.16	0.33±0.026	0.20±0.023
10 mg/kg	1.30±0.056	0.52±0.014	0.13±0.014
30 mg/kg	0.82±0.13	0.47±0.024	0.17±0.009

TABLE II Noradrenaline content µg/g (M±SEM, n=3—16) in the heart, kidneys and brain of guinea pig in controls and after injection of 200/300 mg/kg IMN and MPP intraperitoneally in 1 5 ml saline Organs removed 3—24 hrs after injection as indicated

Time after injection	IMN 200 mg/kg Heart	Kidney	Brain	MPP 300 mg/kg Heart	Kidney	Brain
Control	2 16±0 16	0 33±0 026	0 20±0 023	2 16±0 16	0 33±0 026	0 20±0 023
3	0 60±0 095	0 23±0 017		0 96±0 13	0 24±0 012	0 20±0 012
6	0 37±0 026	0 23±0 019	0 18±0 01	0 68±0 11	0 24±0 025	0 16±0 014
12	0 25±0 020	0 21±0 020	0 17	0 18±0 05	0 22±0 025	0 14±0 015
24	0 92±0 17	0 20±0 17	0 22±0 01	1 23±0 11	0 29±0 012	0 15±0 006

NA content of the kidneys was not as marked as that on the heart, but a statistically significant decrease was nevertheless observed

The effects on the heart of 100 mg/kg were rather marked and with 200 and 300 mg/kg the NA depletion was more longlasting, showing a low level plateau from 6 to 12 hrs Thereafter a slow recovery took place which was not complete, however, in 24 hrs (Fig 2 Table II)

In the kidneys the NA content was significantly lowered after these doses of IMN and MPP but not in the brain

2 *Effect of MPP on the NA content of guinea pig organs*

MPP showed in general the same type of effects as IMN although somewhat higher doses were required to cause depletion Thus 30 mg/kg of MPP had no consistent effect on the NA content of the heart whereas the maximal effects of 300 mg/kg MPP were similar to those obtained with 200 mg/kg of IMN after 12 hrs (Table II) but less marked at 3 and 6 hrs

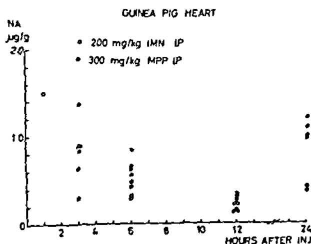


Fig 2 Noradrenaline µg/g in guinea pig heart various times after intraperitoneal administration of IMN (200 mg/kg) and MPP (300 mg/kg)

TABLE III NA content μg/g (M ± SEM n = 6—12) in normally innervated decentralized and denervated rat submaxillary gland various times after IMN 300 mg/kg i p Decentralization 6 days before IMN injection Denervation 6 hrs before NA determination

Control		Time hours after IMN injection					
		1 1/2	3	6	12	18	24
Innervated	0.86 ±0.055	0.53 ±0.036	0.41 ±0.027	0.35 ±0.072	0.26 ±0.041	0.35 ±0.070	0.74 ±0.051
Decentralized	0.83 ±0.055	—	0.59 ±0.086	0.53 ±0.049	0.28 ±0.029	0.33 ±0.051	0.74 ±0.096
Denervated 6 hrs	0.96 ±0.10			0.46 ±0.027			

3 *Denervated and decentralized rat submaxillary gland*

In order to establish firstly whether the reduction of the NA content in organs after administration of IMN depended on an action on the peripheral part of the neuron and secondly whether the nerve impulses normally mediated through the adrenergic nerves were of importance some experiments were carried out on rats in which one submaxillary gland was normally innervated and the second one denervated or decentralized

As shown previously (Benmiloud and Euler 1963) denervation of the rat submaxillary gland causes a gradual depletion of its NA content beginning after 6—8 hrs In agreement with this finding the NA content of the gland 6 hrs after denervation was still in the normal range (0.95 ± 0.10 μg/g) On the other hand the NA content of the 6 hr denervated gland was considerably reduced in the animals which had received IMN at the time of denervation (0.46 ± 0.027 μg/g)

As to the effect of decentralization which by itself does not lower the NA level Table III shows that the NA content falls after IMN to between 1/3 and 1/4 of the control value in 12 hrs in the normally innervated as well as in the decentralized glands However at 3 and 6 hrs after the administration of IMN the NA values were distinctly higher in the decentralized gland indicating a slower release of NA under these conditions At 6 hrs the difference was highly significant

4 *Effect of cold exposure on the NA depleting action of IMN in the rat heart*

Rats were exposed to +2° for various lengths of time and received at the beginning of the cold exposure a dose of 300 mg/kg i p The hearts were excised and analyzed for NA after the exposure

From Table IV it can be seen that even if cold exposure increases adrenergic nerve activity as indicated by the increased urinary excretion of NA (Leduc 1961),

TABLE IV. NA content (μg/g, M±SEM, n=3—10) in the heart of rats kept at room temperature and at +2° C various time intervals after i. p. injection of 300 mg/kg IMN

Control		Time, hours after IMN injection						
		1 1/2	3	6	12	18	24	30
Room temp. (+22°)	0.67 ±0.012	0.47 ±0.049	0.31 ±0.050	0.25 ±0.024	0.18 ±0.054	0.31 ±0.032	0.43 ±0.025	0.47 ±0.011
Cold (+2°)		0.44 ±0.073	0.25 ±0.030		0.23 ±0.032	0.32 ±0.017	0.44 ±0.034	0.45 ±0.01[illegible]

TABLE V. Urinary excretion of NA and A in μg/kg/24 hrs for 3 consecutive days in the untreated rat *after saline 1 ml and after treatment with 50, 100 and 300 mg/kg IMN i. p. daily (M±SEM, n=6)*

	Untreated controls		Saline 1 ml i. p.		IMN					
					50 mg/kg		100 mg/kg		300 mg/kg	
	NA	A	NA	A	NA	A	NA	A	NA	A
Day 1	2.4 ±0.20	0.29 ±0.04	2.8 ±0.18	0.60 ±0.10	3.0 ±0.29	0.64 ±0.06	2.2 ±0.25	0.51 ±0.07	2.4 ±0.30	0.67 ±0.0[illegible]
Day 2	2.3 ±0.09	0.44 ±0.06	3.3 ±0.41	0.37 ±0.14	3.8 ±0.93	0.61 ±0.13	3.3 ±0.61	0.60 ±0.13	4.4 ±0.74	0.65 ±0.14
Day 3	2.3 ±0.19	0.46 ±0.06	3.0 ±0.25	0.42 ±0.09	2.3 ±0.11	0.70 ±0.14	3.2 ±0.35	0.42 ±0.11	4.3 ±0.89	0.47 ±0.12

and the increased turnover of NA (Olivero and Stjarne 1965), there is no significant change in the degree of NA depletion in the rat heart. Recovery of the NA content to normal was not attained 30 hrs after the injection.

5. *Effect of IMN and MPP on catecholamine excretion in urine*

In the study by Westfall (1965) on the action of nicotine and some nicotine derivatives on the catecholamine excretion in urine it was noted that nicotine in a dose of 1 mg/kg i.p. in the rat caused an increase in the excretion of adrenaline (*A*) while the effect on NA was less clear.

Both compounds IMN and MPP have been tested on the excretion of NA and A in the rat in doses of 50, 100 and 300 mg/kg i.p. As seen in Table V IMN caused no marked change in catecholamine excretion even after large doses. A barely significant increase in the NA excretion is seen on the second day for the highest dose of IMN

and in the excretion of A on the first day, when the untreated animals are used as controls No significant differences are observed, however, between the saline treated and the IMN treated animals

The results obtained with MPP were similar, in no instance did significant alterations in the catecholamine excretion occur Neither was any significant change in the VMA excretion observed after the two compounds

6 *Isolated organs*

Rabbit jejunum No effects were recorded with IMN in concentrations up to 1 μg/ml A stimulating effect was observed with 2 5 μg/ml and higher of IMN and MPP

Guinea pig ileum No effects were recorded with IMN concentrations up to 20 μg/ml whereas MPP stimulated the gut in 5 μg/ml and higher

Guinea pig vas deferens IMN and MPP inhibited in concentrations of 1 5—5 μg/ml contractions elicited by field stimulation

Rectus abdominis muscle of frog In concentrations up to 10 μg/ml IMN or MPP did not affect the contractile state of the muscle The contraction elicited by 1 μg/ml of nicotine was not influenced by IMN or MPP in concentrations up to 10 μg/ml

Discussion

Nicotine and dimethylphenylpiperazine (DMPP) are known to cause a release of NA in the perfused heart (Lindmar and Muscholl 1961) Although part of this effect may be due to ganglionic stimulation, the possible stimulating action of nicotine at the terminal parts of adrenergic nerves should be considered (*cf* Ferry 1963, Blakeley, Brown and Ferry 1963, Dhalla 1967) The results obtained with the quaternary nicotine analogue IMN in the present experiments on normally innervated, decentralized and denervated submaxillary glands of the rat seem to indicate that IMN exerts its action on the axonal part of the postsynaptic adrenergic neuron, presumably at the terminal parts, since Armett and Ritchie (1960) found that the nerve endings of C fibres are more sensitive than the axons The NA releasing effect of nicotine-like compounds has been ascribed to depolarization of the axon membrane and is prevented by hexamethonium and low external Ca^{2} (Lindmar, Loffelholz and Muscholl 1967) The present results show that IMN reduces the NA content to about one half of the original amount in the 6 hr denervated submaxillary gland of the rat while it is still normal in the 6 hr denervated control The degree of depletion in the denervated gland is similar to that in the decentralized gland, suggesting that the effect on the cell body of IMN is of less importance than the peripheral effect on the axon The results further suggest that the normally occurring nerve impulses in the adrenergic nerves accelerate the NA loss Thus the NA content in the decentralized gland was significantly higher 3 and 6 hrs after IMN than in the normally innervated gland, indicating a delay in the depleting action when the neuron is not exposed to normal nerve activity

On the other hand an increased impulse frequency, as occurring during exposure to cold, caused no significant action on the NA content of the rat heart

Other studies seem to indicate that the compounds also exert an inhibitory action on the reuptake of the transmitter at the axon membrane level (Bhattacharya 1968 Hedqvist 1969) It thus seems conceivable that the compounds tested not only have a direct releasing action on the terminal axon membrane of the adrenergic nerves, thereby causing a leakage of transmitter from the stores which is not wholly compensated for by new synthesis, but also interfere with the normal reuptake of NA

While the effect of the compounds tested is marked on the heart, the action on the brain is small due to the quaternary nature of the compounds

The excretion studies have shown that although the nerve stores of NA in the tissues are markedly depleted by IMN even in moderate doses, there is only a tendency towards increased output of free NA and A in urine This is also the case when nicotine itself is given

The slowly appearing effect of IMN on the NA content of the guinea pig heart in doses 20—40 times higher than those of nicotine needed for similar effects may explain why IMN lacks an acute action on isolated organs in doses 500 times those of nicotine The general reactions in the animals were also moderate, consisting in certain signs of excitation and unrest together with hyperpnea and increased heart frequency particularly after the higher doses The possibility should perhaps be considered that the action is due to a metabolite of IMN or MPP rather than to the compound itself Under certain conditions, however an acute NA releasing effect of the two compounds can be clearly demonstrated (Euler and Persson 1969)

The results presented here gain further interest in view of the possible formation of IMN itself as a metabolic product of nicotine (McKennis Turnbull and Bowman 1963)

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Uptake of Some Catecholamines and Their Precursors into Neurons of the Rat Ciliary Ganglion

By

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Abstract

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No adrenergic perikarya and only vascular adrenergic terminals were found in ciliary ganglia of normal rats, cats, guinea pigs goats and monkeys but adrenergic neurons occurred in animals that died under severe stress Small, intensely fluorescent cells were irregularly observed in rats and cats and in a cebus monkey In rats Nialamide and L DOPA (but not D DOPA) induced specific fluorescence in certain neurons which was prevented by dopa *decarboxylase inhibition* a *Methyldopamine and a methyldopa also induced fluorescence,* but less effectively The increase in fluorescence in the ciliary ganglion was always less than in the superior cervical ganglion Cervical sympathectomy did not influence the appearance of fluorescence in the ciliary ganglion The results were interpreted to indicate that the ciliary ganglion contains a system of neurons which are presumably intraganglionic do not normally contain catecholamines in sufficient concentration to make them readily demonstrable presumably contain dopa decarboxylase and monoamine oxidase and can take up certain amines These neurons are perhaps ordinary adrenergic nerves operating with an unusually low concentration of noradrenaline but it cannot be excluded that they have a transmitter substance that is not noradrenaline

According to classical concepts the ciliary ganglion is a structure belonging to the parasympathetic nerve system It has long been thought to contain only cholinergic nerve cells and presynaptic terminals (Koelle 1955 Giacobini 1959 Koelle and Koelle 1959 Taxi 1961) Attempts have been made to classify the ganglion cells into several categories according to their appearance after silver staining (*e g* Pines 1927, Kurus 1956), but this classification has not so far proved to be of any functional significance

However, more recent work has created doubts whether the ciliary ganglion is a purely cholinergic structure Hamberger Norberg and Ungerstedt (1965) noted adrenergic terminals around some perikarya of the feline ciliary ganglion Hurkun (1966) also noted adrenergic terminals in the ciliary ganglion of rats together with some few perikarya with weak fluorescence suggesting them to be adrenergic However, pretreatment with high doses of noradrenaline was necessary to demonstrate

these terminal with certainty In birds, the ciliary ganglion regularly contains adrenergic terminals, particularly in the part containing the small ganglion cells (Ehinger 1967) In cynomolgus monkeys perikarya and terminals displaying a typical catecholamine fluorescence occur upon injections with L-DOPA, when the animals are pretreated with a monoamine oxidase inhibitor (Ehinger 1966) In connexion with studies on release and uptake mechanisms of catecholamines in shock, it was unexpectedly found that the ciliary ganglion in slowly exsanguinated dogs contained a striking number of varicose terminals and perikarya which displayed a specific catecholamine fluorescence

Obviously, these new observations conflict with the concept of the ciliary ganglion as a purely cholinergic structure On the other hand the catecholamine fluorescence seen in the mammalian ganglion appears only under circumstances not usually necessary for the demonstration of the catecholamines in ordinary adrenergic nerves These discrepancies initiated the present study on the uptake of various monoamines and their immediate precursors into nervous structures of the ciliary ganglion

Materials and Methods

The main study was performed on white rats (Anticimex, Sweden) Normally the animals were killed by a massive overdose of ether or by a blow on the head Since the ciliary ganglia are too small to be dissected out directly with sufficient speed, a partial dissection of the excised orbital content was performed resulting in a tissue mass mainly of nerves and some muscular and connective tissue This tissue piece was freeze-dried and processed for fluorescence ... (see Falck and ... serial sectioning

... injected intra ... which was dis- ... the substances were dissolved in 0.9 % NaCl

Nialamide (Pfizer Ltd) 100 mg/kg 3 hrs (4 rats)

... (4 rats each)

... rats each)

... rats each)

... AB Ferrosan Malmo

... sympathetic chain. The ... the animals were given ... and L-DOPA (100 mg/kg 1 hr)

For comparison the superior cervical ganglion was studied in animals treated with the following drugs

Reserpine 2 mg/kg 24 hrs + Nialamide 100 mg/kg 3 hrs (3 rats)

Reserpine 2 mg/kg 24 hrs + Nialamide 100 mg/kg 3 hrs + L-DOPA 20 40 or 60 mg/kg 1 hr

...

squirrel monkeys 4 cynomolgus monkey... as rapidly as possible with an overdos... in light barbiturate anesthesia, their ... intravenous infusion of dextran ...



Fig 1 Fluorescence and phase contrast micrograph of the ciliary ganglion normal rat There is no specific fluorescence visible in the ganglion cells nor are there any adrenergic terminals ×290

Results

Adrenergic terminals were only rarely encountered in the ciliary ganglia of either species studied if the animal had been rapidly killed These terminals followed small blood vessels in most cases if not all Normally, fluorescent perikarya did not appear in these animals (Fig 1) In the slowly exsanguinated dogs, however, numerous adrenergic terminals as well as perikarya appeared, exhibiting a strong formaldehyde induced fluorescence (Fig 2) In 3 rats unintentionally killed slowly (about 5 min) with ether in a closed glass jar, a weak fluorescence was also observed in some perikarya together with some adrenergic terminals around nerve cells (Fig 3), but this was not consistently reproducible

Groups of so-called small intensely fluorescent cells (SIF cells Norberg Ritzen and Ungerstedt 1966) usually provided with only short tapering processes were observed in the ganglia of rats, of cats and of a cebus monkey At times the processes of these cells were seen to form baskets around non fluorescent perikarya (Fig 4) but this was not a regular feature Nor were these strongly fluorescent cells regularly seen in the ganglia despite these always being serially sectioned At times SIF cells were seen in nerve trunks connected to the ciliary ganglion

Fig 2 Fluorescence micrograph of the ciliary ganglion of a dog that died from haemorrhagic shock The ganglion contains numerous fluorescent perikarya and terminals ×95



Fig 3 Ciliary ganglion rat unintentionally killed slowly There are many weakly fluorescent terminals *in the ganglion* and one weakly fluorescent perikaryon At the bottom a vascular adrenergic nerve fibre with ordinary fluorescent intensity ×260

Fig 4 So-called SIF cells in the ciliary ganglion of a cebus monkey The processes of the SIF cells can be seen to form a basket of presumably synaptic character around a nerve cell ×200

Fig 5 Ciliary ganglion rat treated with Nialamide and L-DOPA There are several fluorescent perikarya Fluorescent terminals lie close to both fluorescent and non fluorescent nerve cells ×480

Fig 6 Ciliary ganglion of a rat treated with a methylnordarenaline Thei terminals in the *ganglion* ×*440*



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Fig 2 Fluorescence micrograph of the ciliary ganglion of a dog that died from haemorrhagic shock The ganglion contains numerous fluorescent perikarya and terminals ×95

Discussion

It is clear from this study that the normal mammalian ciliary ganglion does not contain perikarya with a catecholamine concentration high enough to be detected with the fluorescence microscope Estimates have shown that the concentration of catecholamines is below 10 μg/g when no fluorescence can be detected in 5—10 μ sections (Norberg and Hamberger 1964) A few fluorescent terminals may occur scattered in the normal ganglion, but they seem usually to be connected with blood vessels rather than with nerve cells

The intensely fluorescent cells noted in the ganglia and nerve trunks are very similar to the small intensely fluorescent cells found in and at, *e g*, the superior cervical ganglia (Eränkö and Härkönen 1965, Norberg Ritzen and Ungerstedt 1966), in the heart (Jacobowitz 1967, Ehinger *et al* 1968), in male and female internal genitals (Owman and Sjöstrand 1965, Sjoberg 1967), in the pancreas (Alm *et al* 1968), and in several other tissues (Falck and Ehinger, unpublished) They have recently been shown to contain dopamine in several species including rats (Ehinger *et al* 1968, Bjorklund *et al* 1969) Their function is unknown From the morphological appearance (Fig 5), they seem, at least sometimes, to form synapses on ganglion cells *On the other hand their irregular occurrence makes it unlikely* that they are indispensable Although their ultrastructure (Grillo 1966, Williams 1967) is similar to that of nerve cells (Elfvin 1968) it cannot be excluded that they represent aberrant or vestigial neural crest cells similar to the para aortic cells found in sucklings (Lempinen 1964 and 1966)

When rats are treated with a monoamine oxidase inhibitor (Nialamide) and high doses of L-DOPA, a certain proportion of nerve cells of the ciliary ganglion and a number of terminals display specific fluorescence (Fig 6), indicating the presence of, presumably, DOPA or dopamine in them Obviously, these cells must possess a mechanism for the uptake of either or both of these substances A similar mechanism may be presumed to operate in the ciliary ganglion of cynomolgus monkeys and pigeons (Ehinger 1966, 1967)

The facts that the decarboxylase inhibitor NSD 1015 largely impedes the appearance of fluorescent nerve cell bodies, and causes the fluorescence to appear also in the nuclei, suggest that the cells contain the enzyme dopa decarboxylase and that dopamine is the substance mainly responsible for the fluorescence after treatment with Nialamide and L-DOPA The first conclusion is augmented by the observation that D DOPA seems able to enter the nerve cells, none the less no accumulation of fluorescence occurs the dopa decarboxylase is well known to be stereospecific for L-DOPA and no dopamine can therefore be formed and retained That D DOPA can enter the cell is shown by the increased diffuse fluorescence shortly after the injection of the drug

In that the fluorescent neurons of the ciliary ganglion can take up L-DOPA and convert it to dopamine, they resemble other adrenergic neurons (*cf* Malmfors 1965) However, the dose necessary for this phenomenon (≥40 mg/kg) is much higher

than that needed to increase the fluorescence intensity of peripheral adrenergic terminals as shown by the strong fluorescence induced already by L DOPA 20 mg/kg in cervical sympathetic ganglia of reserpinized animals Already in this respect the fluorescent neurons of the ciliary ganglion thus differ from classical adrenergic neurons

It is further well established that adrenergic neurons can take up amines such as *a* methyldopamine noradrenaline, and *a* methylnoradrenaline by means of a pump in the cell membrane (*cf* Malmfors 1965) Fluorescent perikarya and terminals appear in the ciliary ganglion after the injection of these drugs except in the case of *a* methylnoradrenaline when only terminals appear The reason for this difference is not clear but it is to be noted that the animals did not survive more than about 15 min when treated with this drug and that there is then usually only a minor increase in the fluorescence intensity in the superior cervical ganglion Thus presumably *a* methylnoradrenaline was not seen in the cell bodies because the time was too short for sufficient accumulation The effect of the other amines was usually studied after 1 hr, and a strong increase in the fluorescence of the superior cervical ganglion used as control was regularly found The doses needed to produce fluorescence in ciliary ganglion cells however are much higher than those needed to increase the fluorescence intensity (*i e* the amine content) of sympathetic neurons to a corresponding level either in the cervical ganglia or in the peripheral terminals There is thus again a qualitative similarity between the neurons with induced fluorescence in the ciliary ganglion and sympathetic neurons and also a striking quantitative difference the neurons have an uptake mechanism but the drugs inducing fluorescence are taken up less readily in the ciliary ganglion than in ordinary sympathetic neurons

The absence of specific fluorescence in normal animals is of interest in connexion with the observations on the effect of the drugs It could conceivably be due to a very rapid amine turnover in an otherwise ordinary adrenergic neuron where the concentration is thus prevented from reaching visible levels This seems less probable however since the treatment with various monoamine oxidase inhibitors blocking the breakdown to a considerable extent did not result in any appearance of fluorescent perikarya or terminals

The occasional occurrence of perikarya with specific fluorescence in slowly killed rats and the regular appearance of fluorescent perikarya and terminals in does dying of haemorrhagic shock can be understood on the basis of an adrenergic uptake mechanism in these tissue components as suggested above These animals most likely have high concentrations of circulating catecholamines which could thus be taken up into ganglionic structures possessing the uptake mechanism

The fact that the fluorescent terminals of the ciliary ganglion remain after cervical sympathectomy demonstrates that they originate either in the central nervous system or within the ganglion Preganglionic severing of the oculomotor nerve has been performed (Ehinger unpublished) resulting in the disappearance of the fluorescent fibres, but the observation is unreliable because of severe inflammatory

reactions in the ganglion On the other hand the terminals arising from the perikarya made fluorescent with Nialamide and L-DOPA must remain intraganglionic since no fluorescent terminals are visible in any of the orbital tissues when sympathectomized animals are given Nialamide and L DOPA Thus in all probability at least a large proportion of the fluorescent terminals and all the fluorescent perikarya of the ganglion must represent wholly intraganglionic neurons These neurons can be compared with the intraganglionic adrenergic neurons described in some visceral ganglia (Hamberger and Norberg 1965), which however are true adrenergic neurons displaying high fluorescence in normal animals

It thus seems that the ciliary ganglion of rats (and possibly of other species in which fluorescent neurons have irregularly been observed *cf* above) contains a system of neurons characterized by the following criteria they are presumably intraganglionic, they normally do not contain catecholamines in sufficient concentrations to make them readily visible with the method of Falck and Hillarp they presumably contain dopa decarboxylase and monoamine oxidase they can take up amines such as dopamine and *α* methyldopamine although these amines are not as readily taken up as in conventional sympathetic neurons they can be made to contain a fluorogenic substance (presumably mainly dopamine) by treatment with Nialamide and high doses of L DOPA but not with Nialamide and D DOPA finally they can also be made fluorescent with *α* methyldopa although this drug is taken up less readily than by ordinary adrenergic neurons It is obvious that there are nerve cells in the ciliary ganglion that are not cholinergic which is in accordance with the observation of differences in the acetylcholinesterase content of different cells in the ciliary ganglion of rats (Huikuri 1966) The presence of these unconventional neurons readily explains why Ehinger (1964) first failed to find fluorescent structures in the ciliary ganglia of rabbits and guinea pigs and also later was unable to find neurons regularly in rats cats dogs cynomolgus monkeys and goats (Ehinger 1966 and unpublished) Although the neurons of the ciliary ganglion in which fluorescence can be induced resemble sympathetic adrenergic neurons there are obviously enough differences to keep them apart as a different type or variety It may be that they are ordinary adrenergic nerves operating only with unusually low concentrations of noradrenaline Such nerves may occur in the murine pancreas (Alm *et al* 1967) However it cannot be excluded that the unusual neurons of the ciliary ganglion have a transmitter substance that is not noradrenaline In this case it must be supposed that the L DOPA is handled by the cells in a fashion mimicking that of the normal transmitter substance and/or its precursor Such mimicking is known in *e g* the histamine containing cells of the rat stomach (Hakanson and Owman 1967) which handle exogenously administered L DOPA much in the same fashion as do the unusual nerve cells of the ciliary ganglion It is also known that the adrenergic nerves in the pineal gland take up and store 5 hydroxytryptamine from the surrounding pineal cells (Bertler Falck and Owman 1963 In the superior cervical ganglia from rats treated with Nialamide and L DOPA *α* methyldopamine or *α* methyldopa almost all ganglion cells showed specific fluc

the normal ganglion a certain proportion shows little or no such fluorescence Further preliminary observations on the normally non-fluorescent intramural neurons of the rat intestine have shown that some of them have properties similar to the unconventional neurons of the ciliary ganglion It may be that this neuron type is not restricted to the ciliary ganglion alone

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Is There an Interaction between Dopamine and Noradrenaline Containing Neurons in the Brain?

By

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Blockade of the hydroxylation of tyrosine causes a reduction of dopamine (DA) as well as of noradrenaline (NA) in the brain (Spector, Sjoerdsma and Udenfriend 1965) During the last few years tyrosine hydroxylase inhibitors have been extensively used for estimation of the turn-over of brain catecholamines under different conditions (Brodie *et al* 1966 Corrodi and Malmfors 1966) Even though such studies have given useful information, it cannot be excluded that the amine depletion in itself changes nerve activity and turn-over It has been shown that a potent inhibitor of the last step in the synthesis of NA *i e* the hydroxylation of DA was capable of depleting NA more rapidly than a tyrosine hydroxylase inhibitor (*cf* Persson and Waldeck 1969 Goldstein and Nakajima 1967) It occurred to us that these results might possibly be explained by an interaction between DA and NA containing neurons in the brain In order to investigate these problems further the experiments reported below were performed

The inhibitor of tyrosine hydroxylase *a* methyltyrosine methylester (H 44/68) was given to mice 1 hr later followed by the inhibitor of DA β hydroxylase FLA 63 (Carlsson *et al* 1969 *cf* Svensson and Waldeck 1969) Five hours after the first injection the animals were killed and the content of NA in the brain determined as described by Bertler *et al* 1958) Animals receiving either of the two drugs or untreated animals were run in parallel (Table I) The reduction brought about by FLA 63 was greater than that caused by H 44/68 although this drug acted 1 hr longer than did FLA 63 Pretreatment with H 44/68 partially counteracted the FLA 63 induced reduction of NA ($P<0.05$) When evaluating these data it should be kept in mind that the action of FLA 63 started at a time when NA was already somewhat depressed by H 44/68 As indicated by preliminary data an analysis of the time course will reveal a more striking difference

In the next experiment H 44/68 was given together with apomorphine which has been shown to stimulate the DA receptor (Ernst 1967 Anden *et al* 1967) Other animals received either of the drugs and untreated animals served as controls 2 hrs after the injections the animals were killed and NA in the brain was determined (Table II) Apomorphine alone caused a slight though not significant reduction of

TABLE I Effect of H 44/68 and FLA 63 on the level of noradrenaline in the mouse brain H 44/68 200 mg/kg was given i p to mice grouped six by six 1 hr later FLA 63, 40 mg/kg was given i p A reinjection with 20 mg/kg of this drug was made 2 hrs after the first dose The animals were killed 5 hrs after H 44/68 had been given Animals receiving either of the two drugs were run in parallel Untreated animals served as controls

Brain noradrenaline µg g	Control	H 44/68	FLA 63	H 44/68 + FLA 63
Mean	0 41	0 22	0 09	0 12
S E M	0 026	0 024	0 010	0 006
number of groups	13	6	6	6

NA but together with H 44/68 it significantly increased the reduction of NA brought about by this drug ($P<0\ 001$)

It may be concluded that NA disappeared faster after β hydroxylase inhibition alone than after the combined inhibition of tyrosine hydroxylase and β hydroxylase On the other hand, when the DA receptors were stimulated by apomorphine the disappearance of NA after tyrosine hydroxylase inhibition was accelerated These results might be interpreted as follows The activity of the NA containing neurons, and thus the turn over of NA in the brain is biased by the activity of DA containing neurons Thus the partial depletion of DA caused by inhibition of tyrosine hydroxylase inhibits the turnover of NA presumably by inhibiting the impulse flow in the NA neurons Activation of DA receptors should then have the opposite effect This in fact, appears to be the case, judging by the experiment with apomorphine

The possibility of such interrelationships as depicted above should be kept in mind when interpreting turnover data following administration of synthesis inhibitors receptor blocking agents etc While this work was in progress a preliminary note by Goodchild (1969) appeared, in which what appears to be very similar observations were briefly reported in so far as the interaction between tyrosine hydroxylase and DA β hydroxylase inhibitors is concerned

TABLE II Effect of apomorphine and H 44/68 on the level of noradrenaline in the mouse brain Mice grouped six by six received simultaneously apomorphine 25 mg/kg and H 44/68 200 mg/kg i p Apomorphine was reinjected (15 mg/kg) 1 hr after the first dose 2 hrs after the first injection the animals were killed Animals receiving either of the two drugs were run in parallel The control material is identical with that in Table I

Brain noradrenaline µg/g	Control	Apomorph	H 44/68	Apomorph + H 44/68
Mean	0 41	0 34	0 30	0 19
S E M	0 026	0 016	0 012	0 013
number of groups	13	7	6	8

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Effect of Physical Training on Circulation during Prolonged Severe Exercise[1]

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Abstract

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Before and after 22 weeks of physical training, oxygen uptake, heart rate and cardiac output (dye dilution technique) were measured in 7 male subjects (22 to 26 years old) during prolonged severe exercise (60 min on 75 % of the individual's maximal oxygen uptake) and during exhausting work loads of about 5 min duration. Maximal oxygen uptake increased from 3.41 l/min (48.0 ml/kg per min) before to 3.64 l/min (52.0 ml/kg per min) after the training ($p<0.05$). Maximal heart rate decreased from 201 beats/min before to 194 beats/min after the training ($p<0.05$). During the 1 hr work period after the training the stroke volume and mean blood pressure became higher ($p<0.05$) and (a—v)O_2 diff. lower ($p<0.05$) than before the training. From the 5th to the 60th min of the prolonged work the heart rate increased about 20 beats/min before as well as after the training.

It is a well established fact that physical training can improve the cardiovascular function, but most earlier studies in this field have been concentrated on the effect of physical training on circulation during submaximal and maximal work of short duration (Rowell 1962, Ekblom *et al.* 1968, Saltin *et al.* 1968). The aim of the present study was to investigate the effect of physical training on circulation during prolonged severe exercise.

Subjects, procedures and methods

7 clinically healthy, previously untrained men, age between 22 and 26 years, voluntered for this study. Anthropological data can be found in Table I.

The subjects came to the laboratory 4 times before the training started. The first 2 times heart rate on a fixed submaximal work load (900 kpm/min) and maximal oxygen uptake (maximal aerobic power), maximal heart rate, maximal pulmonary ventilation and maximal blood lactate concentration were determined. From these data a work, that loaded the individual's oxygen transporting system at about 75 % of the maximum, was chosen for the 1 hr test in the later experiments with catheters. All works were performed on a mechanically braked bicycle ergometer with the pedal frequency 50 rpm.

[1] A preliminary report was given at the XII Scandinavian Congress of Physiology in Turku 1966.

TABLE I Anthropological data and physiological measurements from exercise studies without

Subject	Age yrs	Height cm	Weight kg		Heart Volume ml	
			B	A	B	A
LH	23	185	78.2	77.8	760	780
TB	25	177	63.7	60.4	670	680
BS	22	178	70.4	69.1	760	790
BF	24	175	80.0	80.5	890	890
HF	26	179	74.6	74.0	700	720
PV	25	177	62.0	60.1	610	600
SS	24	180	71.5	71.3	670	660
Means	24.1	178.7	71.5	70.5	723	731
± SE		1.2	2.6	3.0	34	37
SD		3.2	6.8	7.9	90	97
Pdiff				$p > 0.05$		p 0.05

A few days later the subject came to the laboratory at 8 o'clock in the morning after a light fatfree breakfast. Two catheters, one in the brachial artery and the other in a deep cubital vein, were inserted with the Seldinger technique (Berneus *et al.* 1954) and advanced 20—25 cm proximally from the insertion at the level of the elbow. After at least 1 hr rest, arterial blood samples for determination of blood lactates, hemoglobin concentration, hematocrit and plasma proteins at rest, sitting on the bicycle ergometer, were taken. Thereafter the "75 % work load" was started and continued for 60 min. After a few minutes of rest a maximal work load ("max I") was performed and after another 90 to 120 min of rest a second performance with a maximal work load ("max II") was carried out. At the 5th, 15th, 30th, 45th and 60th min of the "75 % work load" and at the end of the two maximal work loads three consecutive measurements of the cardiac output with simultaneous determination of the oxygen uptake were made. In connection with these determinations the arterial blood pressures were recorded and arterial blood samples were drawn for determination of blood lactates, hemoglobin concentration, hematocrit and plasma protein. The total blood loss during the cardiac output experiments did not in any subject exceed 200 ml. On a fourth day the roentgenological heart volume was determined.

The physical training consisted of outdoor running or pedaling the bicycle ergometer and was carried out 3 times a week for in average 22 weeks (range 18 to 27 weeks). Considering the aim of this study the training program was designed to improve the subject's endurance by distance training, but dash training and interval training were also carried out (for definition and details see Ekblom *et al.* 1968).

After the training the experimental procedure was exactly the same as before the training.

Heart volume was calculated from biplane roentgenograms with the subject in supine position (Larsson and Kjellberg 1948).

Plasma proteins were determined according to the macromethod of Kjeldahl.

Hematocrit was determined in a Hawksly microhematocrit centrifuge.

Hemoglobin concentration was determined on a Beckman B spectrophotometer and read against a calibration curve obtained from van Slyke analyses.

Blood lactate concentration was determined with the modified method of Baker and Summerson (1941). To secure peak concentration values after maximal exercises two or more samples were taken.

ECG was frequently recorded on an Elema electrocardiograph or on a Honeywell Visicorder. At least 15 cardiac cycles were used for calculation of the heart rate.

Expired air was collected in Douglas bags and its volume determined in a balanced spirometer. Air samples were analyzed on a modified Haldane apparatus and the oxygen uptake was calculated from these values. Maximal oxygen uptake (maximal aerobic power) was determined according to the method given by Åstrand and Saltin (1961).

catheters indwelled before (B) and after (A) the training

Maximal Oxygen Uptake l/min		ml/kg per min		Max Heart beats min		Heart rate on 900 kpm/min	
B	A	B	A	B	A	B	A
3.50	3.75	44.8	48.2	181	17[illegible]	131	123
3.39	3.65	53.2	60.4	207	200	159	145
3.65	3.94	51.8	57.0	197	187	159	145
3.60	3.94	4[illegible].0	48.9	211	204	140	138
3.49	3.65	46.8	49.3	19[illegible]	191	1[illegible]4	138
3.18	3.26	51.3	54.2	221	214	190	169
3.08	3.27	43.1	4[illegible].9	193	189	[illegible]	140
3.41	3.64	48.0	52.0	201	194	1[illegible].4	142.6
0.08	0.11	1.[illegible]	2.0	[illegible]	[illegible]	7.0	5.2
0.21	0.28	4.0	[illegible].3	13	13	18.5	13.8
	P 0.05		p<0.05		p 0.05		p<0.05

The methods for determination of the cardiac output (dye dilution technique) and the intraarterial blood pressure have been described and discussed earlier (Ekblom *et al* 1968). The error of the method for determination of the cardiac output was 3.0 % to 3.5 % for submaximal and 3.3 % for maximal exercise (Ekblom *et al* 1968).

Ordinary statistical methods were applied for calculation of mean, standard deviation and standard error of the mean. Student's t test was applied on statistics. Level of significance given is $p<0.05$.

Results

Body weight was on an average 71.5 kg before and 70.5 kg after the training ($p>0.05$). During the training period the roentgenological heart volume increased in 4 subjects and decreased in 2 subjects. The average values were 723 and 731 ml ($p>0.05$) respectively.

Exercise studies without catheter. Individual data are given in Table I.

On the given submaximal work load (900 kpm/min) the heart rate decreased with the training from an average 155 beats/min to 143 beats/min ($p<0.05$).

Maximal oxygen uptake increased in all subjects and averaged 3.41 l/min before and 3.64 l/min after the training ($p<0.05$) corresponding to 48.0 ml/kg per min and 52.0 ml/kg per min (8.3 % increase) respectively.

Maximal heart rate declined in all subjects and averaged 201 beats/min before and 194 beats/min after the training ($p<0.05$).

Maximal pulmonary ventilation was unchanged after as compared with before the training (133.0 l/min and 132.7 l/min respectively).

Maximal peak lactate concentration averaged 15.2 mM and 14.0 mM before and after the training ($p>0.05$) respectively.

Exercise studies with catheters. Individual data are found in Table II. Means are illustrated in Fig. 1–3.

TABLE II Individual data from exercise studies with catheters indwelled

Subject		Oxygen uptake l/min	Pulmonary ventilation l/min	Heart rate beats/min	Cardiac output l/min
1 H	Before training				
	5th min	2.70	106.5	149	18.5
	60th min	2.79	95.9	165	18.8
	Max I	3.50	123.5	181	23.8
	Max II	3.37	116.5	179	22.8
	After training				
	5th min	2.78	66.2	149	21.0
	60th min	3.29	92.3	173	21.1
	Max I	3.64	112.6	17[illegible]	23.1
	Max II	3.75	125.2	174	24.3
1 B	Before training				
	5th min	2.65	70.8	177	18.9
	60th min	2.91	95.7	193	20.4
	Max I	3.22	116.4	203	19.3
	Max II	3.30	117.1	203	19.6
	After training				
	5th min	2.90	89.0	176	22.7
	60th min	2.56	71.5	187	21.8
	Max I	3.27	110.1	19[illegible]	22.1
	Max II	3.29	119.9	191	22.8
	Before training				
	5th min	2.31	70.0	159	19.7
	60th min	2.63	66.4	177	22.2
	Max I	3.32	113.3	197	23.9
	Max II	3.16	11[illegible].4	197	23.4
	After training				
	5th min	2.60	77.8	156	19.9
	60th min	2.19	60.2	172	20.8
	Max I	3.29	113.0	187	23.1
	Max II	3.27	108.5	184	23.0
	Before training				
	5th min	2.2[illegible]	71.3	178	21.7
	60th min	2.60	68.2	191	21.8
	Max I	3.48	148.[illegible]	211	22.6
	Max II	3.[illegible]1	140.2	208	24.2
	After training				
	5th min	2.60	77.8	156	19.9
	60th min	2.43	60.2	172	20.8
	Max I	3.29	113.0	187	23.1
	Max II	3.27	108.5	184	23.0

Stroke volume ml	(a—v)O_2 diff ml/liter	Mean blood pressure mm Hg	Hemoglobin concentration g/100 ml	Hematocrit %	Plasma proteins g/100 ml	Blood lactates mM
124	146	118	15.5	47.0	7.4	8.9
114	148	99	15.3	48.0	7.2	5.5
131	147	121	15.5	48.0	6.2	11.6
128	148	127	15.8	47.5	7.4	13.5
141	133	132	15.6	47.0	7.8	4.2
122	156	117	16.0	47.0	7.8	6.1
133	156	137	16.0	48.0	7.6	7.7
140	154	138	15.2	48.0	7.8	8.5
106	141	121	15.5	47.0	7.4	9.1
102	143	89	15.0	45.0	6.9	11.3
95	167	107	15.6	46.0	6.7	14.5
97	148	114	15.6	47.0	6.0	15.4
129	128	109	15.7	47.5	7.4	9.5
117	117	102	14.5	44.0	6.5	6.1
113	148	110	15.2	45.5	7.3	7.5
119	145	—**	15.0	47.0	7.6	12.2
124	122	112	15.0	45.0	7.9	7.0
125	118	107	14.8	44.5	7.5	7.4
121	139	109	15.0	46.0	7.6	9.4
124	129	116	15.0	46.0	7.7	9.1
128	131	115	14.7	46.5	7.7	2.6
121	120	108	14.8	44.0	7.3	1.9
124	142	120	14.6	45.0	7.4	6.5
125	142	118	14.5	44.5	7.4	9.5
122	116	141	14.8	47.5	7.2	5.7
112	119	125	15.0	49.0	6.9	3.0
107	154	142	15.4	49.5	7.2	13.6
116	145	130	15.8	49.0	8.1	17.3
128	131	115	14.7	44.0	7.3	8.3
121	120	108	14.8	43.5	7.7	6.6
124	142	120	14.6	43.0	6.7	8.3
125	142	118	14.5	44.0	7.7	11.0

Table II Conts

HF	Before training				
	5th min	2 38	68 7	168	17 9
	60th min	2 75	82 2	187	20 4
	Max I	3 23	110 0	194	19 6
	Max II	3 10	104 1	194	23 4
	After training				
	5th min	3 12	86 1	176	22 5
	60th min	2 79	83 9	184	23 1
	Max I	3 19	113 3	191	23 6
	Max II	3 35	130 4	187	23 4
PV	Before training				
	5th min	2 09	60 1	176	16 0
	60th min	2 36	71 3	200	18 2
	Max I	2 95	112 0	209	20 5
	Max II	3 08	127 8	211	20 7
	After training				
	5th min	2 26	63 8	188	17 6
	60th min	2 21	64 7	199	18 3
	Max I	2 75	98 9	204	19 4
	Max II	3 01	105 0	207	21 8
SS	Before training				
	5th min	2 33	71 6	150	20 2
	60th min	2 55	78 0	179	21 6
	Max I	2 82	91 6	184	24 6
	Max II	3 05	112 3	193	25 9
	After training				
	5th min	2 38	63 6	151	23 3
	60th min	2 64	70 0	174	23 1
	Max I	3 12	106 4	184	26 6
	Max II	3 11	110 0	189	26 9

* The individual 15, 30 and 45 min values are available after inquiry to the author

The 1 hr work period

The work load during the prolonged exercise was in average 1030 kpm/min before and 1170 kpm/min after the training, giving an oxygen uptake of 2 57 liters/min before and 2 70 liters/min after the training which corresponded to 75 4 % and 74 2 % respectively of the maximal oxygen uptake determined in the exercise studies without catheters

There was a small and significant increase in the cardiac output between the 5th and the 15th min of work both before (1 2 l/min) and after (0 8 l/min) the training (Fig 1) From the 15th min to the end of the 1 hr work period there was no significant change neither before nor after the training The cardiac output became significant higher at corresponding times during the prolonged work after

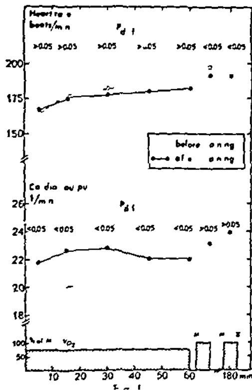
107	133	118	14.4	42.0	7.7	6.6
109	135	86	14.3	43.5	7.2	8.1
101	165	99	15.0	45.0	6.9	11.6
121	132	105	14.6	44.5	7.3	12.5
128	139	126	14.6	45.0	5.8	9.5
126	121	99	13.7	42.[illegible]	5.5	5.2
123	135	110	14.2	44.0	5.5	8.0
125	143	117	14.1	44.0	5.0	13.6
91	131	131	15.4	47.0	8.1	5.7
91	130	106	14.8	46.0	7.9	7.3
98	144	138	15.4	47.5	8.2	8.6
98	149	135	16.1	48.0	8.0	12.7
94	179	177	14.8	48.0	8.3	5.2
92	171	140	14.7	46.5	7.9	3.6
95	142	151	14.5	47.5	7.9	9.2
105	138	159	14.5	46.5	7.9	10.3
135	115	121	15.6	50.0	8.3	5.3
121	118	89	15.2	47.0	8.3	5.7
134	114	116	15.0	46.5	8.5	6.5
134	118	121	15.0	47.5	8.8	11.3
154	109	146	15.1	46.0	7.8	7.5
133	114	124	15.2	45.5	7.7	6.4
145	117	155	15.3	46.5	7.8	9.4
142	116	151	15.3	46.5	8.0	13.2

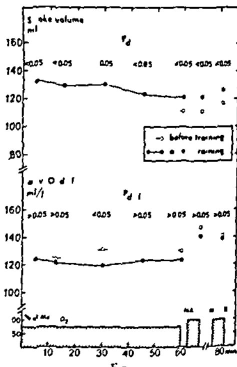
** The blood pressure recording system broke down during the maximal exercise

compared with before the training and averaged for the whole work period 20.0 l/min before and 22.2 l/min after the training.

The $(a-v)O_2$ diff. was unchanged from the 5th to the 60th min of work both before and after the training (Fig. 2). In 5 subjects it averaged lower during the 1 hr work period after compared with before the training, but in 2 subjects (subject HF and BS) it was unchanged. The group mean $(a-v)O_2$ diff. was 129 ml/liter before and 122 ml/liter after the training ($p<0.05$). At corresponding times during the 1 hr work the difference between the pre- and posttraining $(a-v)O_2$ diff. was significant only at the 30th min.

In all subjects the heart rate increased continuously during the 1 hr work both before and after the training. The average heart rate at the 5th, 15th and 60th min


200
175
150
before
26
24
22
20
18
100
50
10
20
30
40
50
60
180 min


ml
160
140
120
100
80
before training
ml/l
160
140
120
100
50
10
20
30
40
50
60

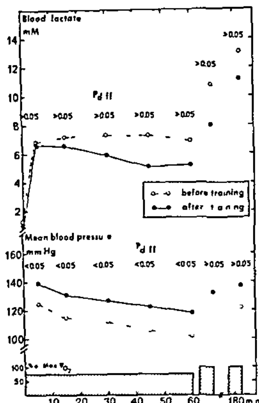


Fig. 3. Blood lactate concentration (n=7) and mean blood pressure (n=6) during the 60 min work period and the maximal exercises before and after the training

Maximal exercise (work time<6 min)

On the maximal exercise performed immediately after the 1 hr test (max I) the work load averaged 1460 kpm/min before and 1680 kpm/min after the training. The work times were 5 min 18 sec and 4 min 30 sec respectively. Total work performed during the maximal exercise was 7740 kpm before and 7560 kpm after the training ($p>0.05$). The oxygen uptake averaged 3.22 l/min both before and after the training.

The cardiac output was 22.0 l/min before and 23.1 l/min after the training ($p>0.05$).

The mean value for the heart rate recorded at maximal exercise was 197 beats/min before and 191 beats/min after the training ($p<0.05$).

The stroke volume was higher after the training and averaged 112 ml before and 121 ml after the training ($p<0.05$).

When the maximal exercise (max II) was repeated after 90 to 120 min of rest the work time was 5 min, 57 sec before the training and 5 min 38 sec after the training (total work 8800 kpm and 9460 kpm ($p>0.05$) respectively).

During the second maximal exercise (max II) oxygen uptake, heart rate, cardiac output, stroke volume and $(a-v)O_2$ diff. were not significantly different

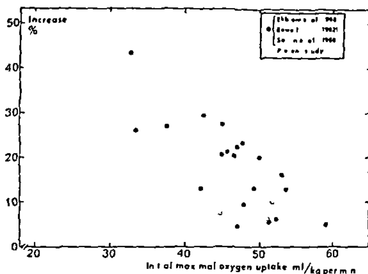


Fig 4 The per cent increase in maximal oxygen uptake after a short term physical training program in relation to the pretraining maximal oxygen uptake in 3 previous studies and the present investigation

from corresponding data obtained in the first maximal exercise (max I) neither before nor after the training (Fig 1 and Fig 2

Maximal blood lactate concentration was higher ($p<0.05$) after the second compared with after the first maximal exercise both before and after the training (see Fig 3

Discussion

It is of importance for the evaluation of the effect of the physical training on circulation during prolonged severe exercise to establish the total effect of the training on the oxygen transporting system

All subjects in this study showed the earlier well documented specific changes in the circulatory response to submaximal and maximal exercise as a result of physical training The function of the oxygen transporting system was improved by 8 3 % as evaluated from the increase in maximal aerobic power (in ml/kg per min) and 10 % if the change in maximal O_2 pulse is considered since the maximal heart rate decreased during the training

In some earlier training studies Robinson and Harmon 1941 Rowell 1962 Ekblom *et al* 1968 Saltin *et al* 1968 a group mean improvement in maximal oxygen uptake at about 15—20 % has been reported and in comparison the 8 % increase in maximal aerobic power after the training in the present study is somewhat low However in the above mentioned earlier investigations there was a wide individual range in the per cent improvement (5 to 43 %) and as seen in Fig 4 it is evident that the pre training value on the maximal oxygen uptake is very important The

highest increase in maximal oxygen uptake in connection with physical training is noticed in subjects with the lowest initial value

There are some indications that the subjects, used in the present study were more fit before the training started than the subjects used in the other training studies mentioned The average pretraining maximal oxygen uptake in this study was 48 ml/kg per min with no subject less than 42 ml/kg per min Regarding this the increase in maximal aerobic power in our 7 subjects lies within or in the lower limit of, what can be called the 'normal' improvement in maximal oxygen uptake in young male subjects after a short term physical training program

The heart rate on the fixed submaximal work load (900 kpm/min) was lower after the training indicating an increased O_2 pulse and thus improved cardiovascular function The increased O_2 pulse must solely be due to an increased stroke volume as the $(a-v)O_2$ diff was lower after the training

Presently, there is an extensive discussion whether or not the trained individual has a different cardiac output on a given submaximal oxygen uptake than the untrained individual Our own data can not solve the question In the earlier training study from this laboratory (Ekblom *et al* 1968) the cardiac output was lower—*i e* the $(a-v)O_2$ diff was higher—on a given oxygen uptake after training while in the present investigation the opposite situation—that is a lower $(a-v)O_2$ diff after the training—was found Furthermore very divergent results have been reported from both cross sectional (athletes non athletes) studies (Bevegård Holmgren and Jonsson 1962 Hanson and Tabakin 1965 Ekblom and Hermansen 1968) and longitudinal training investigations (Freedman *et al* 1955 Rowell 1962 Frick Konttinen and Sarajas 1963, Tabakin Hanson and Levy 1965 Andrew Gusman and Bechlake 1966 Ekblom *et al* 1968 Saltin *et al* 1968) and therefore the question about the pattern in the cardiac output response to submaximal work after training can not presently be answered

All subjects (except subject LH) did neither before nor after the training obtain the same oxygen uptake during the maximal works in the experiments with catheters as during the maximal work without catheters indwelled The reason for this is not known and in some way strange since the work load set and the work time in the circulatory experiments were adequate for reproducing the maximal oxygen uptake measured in the experiments without catheters Similar observations were made in the 1968 study (Ekblom *et al* 1968)

In the experiments without catheters maximal oxygen uptake increased from 3 41 l/min before to 3 64 l/min after the training Since the heart rate recorded in the maximal exercises with and without catheters were almost identical both before and after the training the increased maximal oxygen uptake may be explained by an increase in both stroke volume (as measured) and $(a-v)O_2$ diff Subject LH may serve as an example In this subject the maximal oxygen uptake in the experiments both with and without catheters increased from 3 50 to 3 75 l/min This was due to both a widening of the $(a-v)O_2$ diff and an increase of the cardiac output Since the heart rate during the maximal work decreased the increased cardiac out

put was caused by an increased stroke volume It is worth mentioning that the change in heart volume in this subject and also for the whole group was very small and within the error of the method for determination of the heart volume All these observations in subject LH are in agreement with corresponding measurements during maximal exercise in other training studies (Rowell 1962, Ekblom *et al* 1968 Saltin *et al* 1968)

Earlier studies have shown that during prolonged exercise there is a continuous increase in heart rate (Christensen 1931) and as the cardiac output remains constant over the work period the stroke volume declines concomitant with the increase in heart rate (Saltin and Stenberg 1964, Ekelund and Holmgren 1964) In this respect the findings in the present study confirm these earlier reported observations This decrease in stroke volume during the prolonged work which might be interpreted as an impaired cardiovascular function, was noticed by Saltin and Stenberg (1964) and Ekelund (1966) even during supine work One might therefore expect that physical training would present such an effect of the prolonged exercise on the circulation However although the physical training in this study resulted in an improved cardiovascular function (see above) it did not alter the pattern in the heart rate and stroke volume response to the prolonged work It is possible that part of the increased heart rate during the 1 hr test might be explained by the blood loss but an increase in heart rate of the same order as in the present study during prolonged exercise has been observed even in experiments without catheters indwelled (Christensen 1931)

The heart rate during the 1 hr test increased as much after as before the training (Fig 1) and as the pattern in the cardiac output response to the prolonged work was unchanged the stroke volume declined as much after as before the training (Fig 2) Indications that there were only minor differences between trained and untrained individuals in this respect were given by both Saltin and Stenberg (1964) and Saltin *et al* (1968) In the trained subject (BE) in the former study there was a slight decrease in cardiac output during the prolonged work in contrary to all the untrained subjects On the other hand the stroke volume decreased more in the trained subject than it did in the untrained subjects In the latter study the heart rate (but not cardiac output) was measured in 5 subjects during prolonged severe exercise (80 % of maximal oxygen uptake) for about 30 min in a control situation after 20 days of bed rest and after 55 days of physical training Although the maximal oxygen uptake varied from 2 43 l/min (after bed rest) to 3 97 l/min (after training) the increase in heart rate during the prolonged work was the same in all the three situations

The prolonged work was subjectively felt much easier after compared with before the training which might be explained by the blood lactate concentration (Fig 3) After 5 min of work the blood lactate concentration was the same before and after the training (6 8 and 6 7 mM respectively) This means that since the oxygen uptake was higher after the training the lactate concentration for a given oxygen uptake was lower after the training But the more interesting observation is that after

this initial production of lactate the group mean blood lactate concentration was unchanged throughout the work period before the training, while after the training it decreased continuously in 6 of the 7 subjects For these 6 subjects the decrease was significant (The whole group mean value declined to 5 2 mM (p>0 05) at the 60th min) In the 7th subject (LH) there was a continuous increase in blood lactate concentration from 4 2 mM at the 5th min to 6 1 mM at the 60th min but during the same time the oxygen uptake increased from 2 78 l/min to 3 29 l/min (The latter value is 88 % of his maximal oxygen uptake) In this special case it can not be excluded, that the work load for some reason increased during the 1 hr exercise which might explain the increasing blood lactate concentration

The above discussed effect of the physical training on the blood lactate concentration in 6 of 7 subjects is very important, since accumulation of lactates may limit the individual to perform prolonged severe exercise Furthermore, the sensation of less physical strain during the prolonged work after the training would certainly have been still more pronounced, if the same absolute work load had been used in the pre training and post-training 1 hr tests

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The Acetylcholine Content and Cholinesterase Activity of the Bull Retractor Penis Muscle

By

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Abstract

KLINGE, E *The acetylcholine content and cholinesterase activity of the bull retractor penis muscle* Acta physiol scand 1970 *78* 159—167

The acetylcholine content of the bull retractor penis muscle, duodenum and urinary bladder was assayed by superfusion of the eserinized frog rectus muscle The capacity of retractor homogenates to split acetyl β methylcholine, butyrylcholine and acetylcholine was estimated with the manometric technique The acetylcholine content of the retractor was of the same order of magnitude as that of the duodenum, but it was twice as high as the amount found in the urinary bladder A moderate activity of acetylcholinesterase and non specific cholin esterase was observed The role of acetylcholine in the function of the efferent nerve fibres running in the bull retractor is discussed So far as the classical definitions are applied it is regarded as unlikely that it serves as a neurohumoral transmitter substance either at ganglionic synapses or at neuroeffector junctions There is as yet no conclusive explanation for the mode of action of acetylcholine in these nerve fibres

It has been demonstrated that such doses of atropine that fully block the effect of vagal stimulation in anesthetized dogs (Anrep and Cybulski 1884 Piotrowski 1894, Henderson and Roepke 1933), rabbits (Langley and Anderson 1895) guinea pigs (Spina 1897) and cats (Goldenberg 1965) do not prevent in these animals the penile erection produced by stimulation of the pelvic nerves (also called sacral para sympathetic nerves or nervi erigentes) Concerning dogs somewhat divergent though not convincing results have been reported (Nikolsky 1879, Bacq 1935) Henderson and Roepke (1933) and Bacq (1935) failed in their experiments with dogs to detect acetylcholine (ACh) in the penile perfusate following erection produced by stimulation of the pelvic nerves According to these findings it seems well established that the classical conception of neurohumoral transmission generally attributed to the parasympathetic nervous system cannot as such be applied to the pelvic nerves so far as the erection phenomenon is considered

In all mammalian species having a retractor penis muscle the penile erection probably is accompanied by a simultaneous relaxation of this muscle By stimulat of the pelvic nerves this situ

ley and Anderson 1895) It is conceivable that the relaxation of the retractor muscle is uninfluenced by atropine identically with the simultaneous erection of the penis.

Recently it has been shown with histochemical methods (Klinge, Pohto and Solatunturi 1970) that the nerves of the bull retractor penis muscle contain acetylcholinesterase (AChE) Due to the presence of AChE it was assumed that also ACh would be found The present work was undertaken in order to justify this assumption and to make a quantitative study of the cholinesterase activity in this muscle. The ACh content of some tissues known to contain a parasympathetic innervation in most mammalian species was also determined

Material and methods

I *Acetylcholine*

Following extraction the ACh content of the tissues was estimated on the eserinized frog rectus abdominis muscle The various steps were as follows

Dissection of samples The bulls were of the Ayrshire breed and weighed 140—250 kg The samples were dissected immediately after death of the animals and transported to the laboratory at +2° C The retractor penis muscle was freed from the surrounding fascia and a sample was taken from the proximal middle and distal part The specimens of the duodenum were cut about 50 cm from the pyloric part of the abomasum and those of the urinary bladder from the fundus vesicae urinalis The duodenal and bladder samples comprised all the layers of the respective organ.

Preparation of extracts Both the bound and free ACh were extracted most of the principles applied at this procedure were the [illegible] were the same whether the tissues were cu [illegible] Merck AG the specimens were [illegible] into 3 ml of the extraction med [illegible] 0 14 $CaCl_2$ 0 12 NaH_2PO_4 0 01, and physostigmine base 0 01 The pH of the [illegible] adjusted to 4 with 0 1 N HCl because the stability of ACh is best at this level of acidity (Abdon and Ljunedahl-Ostberg 1944) The samples were heated in a boiling water bath for 5 min After subsequent cooling and centrifugation the supernatants were either assayed for their ACh content or stored overnight at −20 C To prevent hydrolysis of ACh the samples were not neutralized until immediately before the bioassay during which they as well as the standard solutions were kept in ice

Bioassay The superfusion technique of Gaddum (1953) with the modification of Ahmed and Taylor 1957 for the frog rectus muscle was used Before suspension both rectus muscles [illegible] 1952 In addition [illegible] ed su[illegible] +23° C [illegible] as in[illegible] rupted 15 sec before applying an experimental or a standard solution was begun. Both solutions were in volumes of 0 2 ml successively directed on the surface of the muscle at intervals of 8 5 min The duration of an application was 30 sec The contact time counted from the beginning of the application was 90 sec whereupon the superfusion was restarted. The contractions were recorded in a tenfold magnification using a frontal lever and a tension of 2 g Relaxation of the muscle occurred within 3 to 5 min The sensitivity of the preparations varied moderately with different frogs Fig 1 but the dose response was as a rule linear within the range of 10 to 40 ng and remained in most cases unchanged during more than 50 stimulations

Identification of acetylcholine The contractions of the frog rectus caused by the samples were judged to be due to ACh on the basis of the following criteria

1) The samples showed the same dose response relationship and were inhibited by d-tubocurarine (Fig 2 and potentiated by physostigmine to the same degree as the ACh standards.
2) After boiling for 5 min at pH 9 and subsequent neutralization their power to contract the frog rectus was fully abolished
3) On the guinea-pig ileum suspended in Tyrode solution their dose response curve was identical with that of the ACh standards

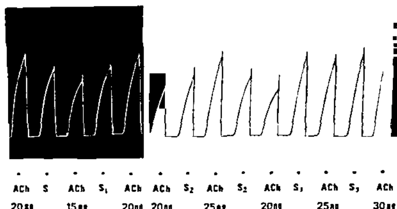


Fig 1 Effect of samples (S) of the bull retractor penis muscle and of acetylcholine (ACh) standards on the superfused rectus abdominis muscle of two frogs

According to Whittaker (1963) it is most likely that the contractions were caused only by ACh though it can not be definitely excluded that some substance chemically closely related to ACh is involved

II *Cholinesterases*

The cholinesterase activity was determined with the manometric technique measuring the CO_2 produced at the hydrolysis of various choline esters The principles applied in this procedure were mainly those described by Augustinsson (1948)

Enzyme preparations The samples of the retractor muscle were obtained in the same way as at the estimation of the ACh content They were ground with 15 volumes (g/ml) of Augustinsson s bicarbonate buffer solution (R_{30}) in a mortar using purified sea sand because about 50 % of the activity of the non specific cholinesterase (nsChE) was destroyed if an Ultra Turrax homogenizer was employed A volume of 0 9 ml of this enzyme suspension was pipetted into the main part of the reaction bottles

Substrates MeCh (d l acetyl β methylcholine chloride Koch Light Laboratories Ltd) was used as a substrate for AChE The substrate used for the nsChE was BuCh (butyrylcholine iodide puriss Fluka AG) The hydrolysis of ACh (acetylcholine chloride crystalline Sigma Chemical Company) which is brought about by both of these enzymes was also measured The rate of hydrolysis of each substrate was examined at a 0 003 0 01 and 0 03 M final concentration of the respective base With all substrates the reaction velocity was highest and remained constant for 80 min when the 0 03 M concentration was used Therefore this concentration was employed in all the experiments and 0 1 ml of a 0 3 M substrate solution was pipetted into the side bulb of the reaction bottles If BuCh was used in the double concentration the velocity of the hydrolysis remained unaltered

Incubation procedure [illegible]
with a side bulb were use [illegible]
80 min were performed [illegible]
volume of the reaction mi [illegible]
mixture containing 95 % [illegible]
vals The spontaneous hydrolysis and the changes in temperature were taken into consideration

[illegible] mmoniumphenyl/
elective inhibitor
ight Laboratories
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ation They were
pipetted in a volume of 0 1 ml into the main part of the reaction bottles before starting the pre incubation Neither the volume of the reaction mixture nor the final concentration of the enzyme were changed since correspondingly more concentrated homogenates were employed

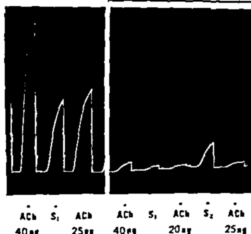


Fig 2 Effect of acetylcholine (ACh) standards and of samples (S) of the bull retractor penis muscle on the frog rectus before and after addition of d tubocurarine to the superfusion fluid

Accuracy of the method All measurements were made in duplicate and the values obtained did not differ more than 4 %. At the incubation of BuCh under the present conditions with the brain homogenates of two adult Sprague Dawley rats, CO_2 was yielded at an average rate of 336 μl/g of fresh tissue/hr. This is about the same as the value obtained by Ord and Thompson (1950) with a similar method.

Results

Acetylcholine The ACh content of various parts of the retractor penis muscle and of duodenum and urinary bladder of the bull is seen in Table I. The quantum of ACh found in these tissues did not show any striking differences. The largest amount was observed in the proximal end of the retractor muscle and in the duodenum and the lowest one in the urinary bladder. In the retractor the ACh content decreased towards the distal end.

Cholinesterases The capacity of different parts of the bull retractor to hydrolyze certain choline esters under specific conditions is presented in Table II. Every part of the muscle splits BuCh at a higher rate than MeCh and ACh is hydrolyzed at a still higher rate than BuCh. Both the AChE and nsChE activities are about as high in the proximal and middle part of the muscle and decrease towards the end.

TABLE I The acetylcholine content in μg/g ± S.D. of some tissues of the bull assayed by superfusion of the eserinized frog rectus abdominis muscle. The figures in parentheses refer to the number of test animals

Retractor penis muscle			Duodenum	Urinary bladder
proximal part	middle part	distal part		
1.6 ± 0.33 (10)	1.0 ± 0.1[illegible] (10)	0.69 ± 0.17 (10)	1.4 ± 0.22 (4)	0.63 ± 0.0[illegible] (4)

TABLE II. Rate of hydrolysis of some choline esters during incubation with homogenates from different parts of the retractor penis muscle of 6 to 8 bulls. The manometric technique was used and the amount of CO_2 evolved is expressed in μl/g of fresh tissue/hr ± S.D.

Part of muscle	MeCh	BuCh	ACh
Proximal	530±75	910±200	1080±190
Middle	570±35	800±220	1140±150
Distal	350±65	560±100	600±120

BW 284 C 51 prevented in the 1×10^{-6} M concentration the hydrolysis of MeCh by 61 % and in the 1×10^{-5} M concentration by 96 %. The same concentrations of IsoOMPA prevented the hydrolysis of BuCh by 33 % and 93 % and those of physostigmine prevented the hydrolysis of ACh by 50 % and 95 %, respectively.

Discussion

I. *The retractor penis muscle*

The amount of ACh detected during the present study in the bull retractor is clearly higher than that previously found in the corpus cavernosum urethrae of the same species (Penttila and Vartiainen 1964). In both of these tissues the physiological role of ACh is as yet undefined. But the existence of such a role is strengthened by the associated presence of AChE, which has been demonstrated also in the corpus cavernosum urethrae (Klinge and Penttila 1969). The tentative assumption can be made that in a tissue like the mammalian retractor penis muscle the location of ACh corresponds to that of AChE (*Koelle 1963, Jacobowitz and Koelle 1965*). In the bull retractor the AChE is confined to the nerve fibres (Klinge, Pohto and Solatunturi 1970) and accordingly this applies also to the location of ACh. The possibility to explain the physiological significance of ACh in these nerves by means of the validity of some of the most important generally known theories will be considered.

Acetylcholine as a neurohumoral transmitter substance. There is no reason to believe that ACh acts as a transmitter substance at ganglionic synapses in the bull retractor. This view is supported by the finding that nicotine affects the isolated muscle only in very high concentrations and its effect is not prevented by hexamethonium (Klinge 1967). Further, nothing that would resemble autonomic ganglia could be detected in the muscle with histochemical methods (Klinge, Pohto and Solatunturi 1970).

In the introduction of this paper attention was called to the results of certain earlier experiments according to which it is unlikely that ACh acts as a transmitter substance at the postganglionic terminals of the pelvic nerves in the dog retractor. For obvious reasons those experiments were not performed with bulls. But there is no support to the statement that ACh would have such a transmitter function at the

respective nerve terminals in the bull retractor, either. The isolated retractor muscles of both animals are poorly affected by ACh. In experiments with the dog retractor Dale (1914) failed to observe any response to this ester, whereas Ludueña and Grigas (1966) obtained some contractions with large doses. Only by using exceptionally high concentrations of ACh could some irregular contractions be produced in the bull retractor (Klinge 1967). The contracted retractor of the dog is not relaxed by ACh (Ludueña and Grigas 1966), and neither is that of the bull (Klinge 1970b).

Acetylcholine and the release of noradrenaline or some other transmitter substance. According to Burn and Rand (1959, 1965) ACh brings about the release of noradrenaline from sympathetic nerve terminals. The conception of these investigators is that in sympathetic nerves ACh is located either in the same fibres as noradrenaline or in adjacent fibres arising from the same ganglion. In both cases it should be able to cause the release of noradrenaline upon stimulation of the sympathetic nerve. A very dense sympathetic innervation was observed in the bull retractor by Klinge, Pohto and Solatunturi (1970). These authors also report that the concentration of noradrenaline is highest towards the end of the sympathetic nerve fibres and decreases sharply in the central direction, whereas AChE probably is present along the whole postganglionic nerve fibre and increases in the central direction. By light microscopic studies it was not possible to decide whether AChE and noradrenaline are located in the same or in concomitant axons. But by no means is there a full correspondence between their locations. The question also remains open whether the bull retractor contains two different types or only one type of efferent nerve fibre.

Stimulation of the appropriate *sympathetic nerves* constantly causes contraction in the dog retractor and this effect is prevented by ergot (Dale 1906). The view that this contraction is entirely caused by noradrenaline may to some extent be supported by the observation that the bull retractor contains large amounts of noradrenaline but probably no adrenaline (Klinge 1970a). So far the possibility has not been excluded that ACh contributes to the release of noradrenaline at sympathetic terminals in the dog retractor. This applies also to the bull retractor, i.e. the theory of Burn and Rand could be valid within certain limits. However, the present knowledge concerning the sympathetic nerves of this muscle is insufficient to justify the theory or to reject it. Some evidence to evaluate the accuracy of the cholinergic link hypothesis will probably be provided by electron microscopy, by studies of the release mechanism in isolated noradrenaline containing nerve granules and by investigations performed with living bulls.

Burn and Rand regard the releasing effect of ACh as a nicotinic one. It is known that atropine does not block the inhibitory effect on the dog retractor produced by stimulation of the *pelvic nerves*. Nevertheless, this can hardly be considered as an indirect support to the concept of Burn and Rand since the pelvic nerves are not known to release noradrenaline. To the author's knowledge there is no report of a successful demonstration of the release of some other transmitter substance effected

by ACh or without it In fact it seems to be completely unknown what happens at the nerve terminals in the retractor penis muscle of any mammalian species upon stimulation of the pelvic nerves

Acetylcholine and axonal conduction The view that ACh is essential for the propagation of nerve impulses is defended by Nachmansohn (1963) According to him ACh controls ion movements by interfering with processes in axonal and synaptic membranes Light microscopic studies suggest the presence of AChE along the whole postganglionic nerve fibres running in the bull retractor This might be regarded as indirect support to the proposition of Nachmansohn if it were generally accepted that AChE is involved in the generation of the bioelectric currents that propagate nerve impulses But there are controversial opinions (*e g*, Hebb 1963 Koelle 1963) Concerning the nerve fibres in the bull retractor the data so far available do not render it possible to defend this theory Neither can its eventual validity be excluded

The presence of homologues of acetylcholine The dose response curves obtained with the samples on the guinea pig ileum were consistent with those of ACh This makes the occurrence of propionylcholine or butyrylcholine as well as that of several other choline esters most unlikely (Whittaker 1963) On the other hand the spleen of the ox should be the only mammalian tissue in which the presence of an ester of choline other than ACh has been demonstrated (Banister Whittaker and Wijesundera 1953 Henschler 1957, Keyl 1957 Whittaker 1963) The ester in question is propionylcholine but its physiological significance remains obscure

In the present study BuCh was hydrolyzed by the bull retractor and a light microscopic study of this muscle indicates that in or near some nerve terminals the nsChE activity is higher than in the surrounding tissue (Klinge Pohto and Solatunturi 1970) The physiological significance of this finding is uncertain and this applies also to the associated presence of a homologue of ACh Certain parts of the ox brain shows a distinct capacity to split BuCh though it is lower than that of the same parts of several other mammalian species (Bernsohn and Possley 1958) But using paperchromatographic separation no ester of choline other than ACh could be detected in the ox brain (Henschler 1956 Keyl 1957) The possible role of such an ester in the function of the nerves in the bull retractor will be considered in some more detail in a further report

II *The urinary bladder and the duodenum*

One of the purposes of the determination of the ACh content of these tissues was to have some rough quantitative control of the accurary of the method The amount of ACh found in the duodenum of the bull was clearly higher than that observed in the ox intestine by Keyl (1957) But it was of the same order of magnitude as the quantity found in the small intestine of the horse and the dog by Chang and Gaddum (1933) These investigators reported also an ACh content of the dog bladder which was about twofold that found by the author in the bladder of the bull It is generally accepted that one of the functions of ACh both in the bladder and

in the duodenum of most mammalian species is to serve as a neurohumoral transmitter substance But according to the present knowledge it is unlikely that ACh transmits either excitatory or inhibitory nervous impulses to the fibres of the retractor penis muscle

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Catechol-O-Methyl Transferase and Monoamine Oxidase Activity in Some Tissues of the Bull

By

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Abstract

KLINGE, E. *Catechol-O-methyl transferase and monoamine oxidase activity in some tissues of the bull.* Acta physiol. scand. 1970. 78. 168—173.

The catechol-O-methyl transferase activity in the retractor penis muscle, liver and kidney of the bull was determined by measuring the amount of metanephrine formed from adrenaline when S-adenosylmethionine was used as methyl donor. The monoamine oxidase activity in the same tissues was estimated by measuring the rate of disappearance of 5-hydroxytryptamine added to the incubation mixture. There was a very low, if any, catechol-O-methyl transferase activity in the retractor muscle, a slight but distinct activity in the kidney and a high one in the liver. The monoamine oxidase activity exhibited by the retractor muscle was moderate. In the liver the activity of this enzyme was high but it was still clearly exceeded by that in the kidney. The role of both enzymes in the function of the retractor muscle is briefly discussed.

The retractor penis muscle of the dog and cat is powerfully contracted on stimulation of the lumbar sympathetic chain (Langley and Anderson 1895). It has recently been demonstrated that in the retractor penis of the bull there is a dense adrenergic innervation (Klinge, Pohto and Solatunturi 1969, Klinge 1969a) and isolated strips of this muscle are vigorously contracted by adrenaline and noradrenaline (Klinge 1967, 1969b). There is certain evidence to assume that the almost continuous contraction of the retractor penis muscle of the bull and of several other mammalian species is maintained by impulses conducted along sympathetic nerves [illegible] involved. Due to this un-

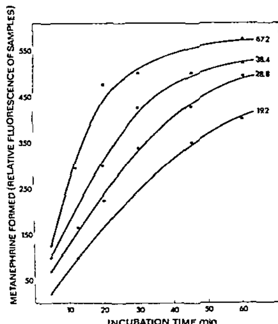


Fig 1 Rate of O methylation of 0 3 µmole of adrenaline by four different amounts of fresh bull liver which are expressed in mg by the figures pertaining to the curves The other reaction conditions were constant and are described in the text Each point represents the mean of three incubations

formed according to Axelrod Albers and Clemente (1959) and the enzyme assay according to Axelrod and Tomchick (1958) with slight modifications as follows

Preparation of extracts The bulls were of the Ayrshire breed and weighed 140—250 kg Samples from the proximal middle and distal part of the retractor muscle and from various parts of the liver and kidney were dissected immediately after death of the animals The renal samples contained about an equal amount of cortex and pulpa The tissues were homogenized in 4 volumes (g/ml) of 0 92 % KCl and centrifuged at 15 000 g for 20 min An Ultra Turrax homogenizer was used for the retractor samples and a glass one for the other tissues Every step was carried out at +2° C

Enzyme assay Various dilutions of the supernatant were made and 0 45 ml of each was preincubated for 5 min with 0 1 ml of 0 1 M $MgCl_2$ and 0 25 ml of 0 2 M phosphate buffer pH 7 8 whereafter 0 1 ml of 3×10^{-3} M adrenaline and 0 1 ml of 1×10^{-3} M S-adenosylmethionine (Sigma Chemical Company) were added Under these conditions the most effective O methylation [illegible] to 28 8 mg of f[illegible] 15 min the rea[illegible] cal shaking for [illegible] ide containing 2 [illegible] ent was equally shaken with 1 5 ml of 0 1 N HCl from which the metanephrine was measured spectrophotofluorometrically at 285/335 nm Known amounts of synthetic metanephrine were always carried through the extraction procedure The recovery was on the average 62 % ranging from 49 to 75 % The results are corrected for the respective recoveries A 1×10^{-4} M concentration of pyrogallol inhibited 80 % of the O methylation of adrenaline effected by the liver and a 1×10^{-3} M concentration 96 %

II *Monoamine oxidase*

The MAO activity of the tissues was determined by measuring the rate of disappearance of 5 hydroxytryptamine (5 HT) from the incubation mixture at 37° C Sjoerdsma *et al* 1955) The incubation technique was a slight modification of those described by Bogdanski *et al* (1957) and Karki *et al* (1962) The extraction and measuring of the remaining 5 HT was performed essentially according to Bogdanski *et al* (1956)

Incubation procedure The retractor samples were homogenized in a cold mortar in 10 volumes (g/ml) of distilled water A mixture containing 1 0 ml of the homogenate, 0 3 ml of

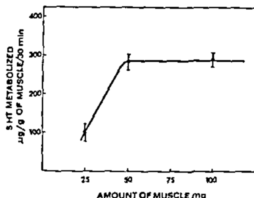


Fig. 2

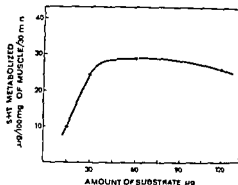


Fig. 3

Fig. 2. Rate of oxidative deamination of 60 µg of 5-HT by various amounts of fresh bull retractor penis muscle under the conditions described in the text. Each point represents the mean of three determinations.

Fig. 3. Rate of oxidative deamination of various amounts of 5-HT effected by 100 mg of fresh bull retractor penis muscle under the conditions described in the text.

0.3 M phosphate buffer pH 7.4 and 1.1 ml of water was preincubated for 20 min whereafter 60 µg of 5-HT in 0.6 ml of water were added to the samples which yielded a reaction volume of 3.0 ml. The incubation time was 30 min during which the reaction rate was constant. Immediately after stopping of the incubation 5-HT was added to the internal standards in the same way as to the samples earlier. It was checked that under the present conditions the highest reaction velocity was achieved by using 60 µg of 5-HT and an amount of enzyme corresponding to 100 mg of fresh retractor muscle (Fig. 2 and 3). The amount of 5-HT metabolized was in linear correlation to the amount of muscle (Fig. 4). With 100 mg of liver or kidney the maximal amount of oxidative deamination was reached when 600 µg of 5-HT used.

Extraction and assay of remaining 5-HT. The incubation mixtures were transferred into bottles containing 4 g of NaCl and 15 ml of butanol into which the 5-HT was extracted by mechanical shaking for 15 min. After centrifugation 10 ml of the butanol phase were equally shaken with 20 ml of heptane and 2.0 ml of 0.1 N HCl from which the 5-HT was measured spectrophotofluorometrically at 300/540 nm. The 5-HT remaining in the samples was calculated in relation to that remaining in the internal standards. The washings with borate buffer were omitted because it was observed that they did not influence the results.

Accuracy and specificity. All measurements were made in duplicate. The values obtained did not differ more than 7 %. A 1 × 10^-[illegible] M concentration of tranylcypromine sulphate (Smith Kline & French Laboratories) totally inhibited the breakdown of 5-HT by the retractor homogenates. A 1×10^{-4} M concentration blocked 95 % of the MAO activity of the liver ho-

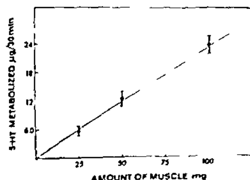


Fig. 4. Correlation between the rate of oxidative deamination of 60 µg of 5-HT and the amount of fresh bull retractor penis muscle under the conditions described in the text.

TABLE I Catechol O methyl transferase activity in some organs of the bull expressed in μmoles of metanephrine formed/g of fresh tissue/hr (mean ± S D) Number of test animals in parentheses

	Retractor penis muscle	Kidney	Liver
	<0 05 (10)	0 12±0 08 (5)	2 9±0 22 (5)

mogenate and 96 % of that of the kidney homogenate A 1×10^{-4} M concentration of semicarbazide hydrochloride (E Merck AG) did not affect the breakdown of 5-HT by the retractor or liver homogenates but it inhibited 79 % of the activity of the kidney homogenate

Results

Catechol O methyl transferase The COMT activity of the tissues is presented in Table I No certain activity was observed in any part of the retractor muscle Thus if there is any activity in this muscle it is below that detectable with the present method i e less than 0 05 μmole of methanephrine was formed/g of fresh tissue/hour The kidney showed a low but distinct activity In contrast to the retractor and kidney the liver displayed a high activity with but little variation between different bulls

Monoamine oxidase The MAO activity is stated in Table II In all parts of the retractor muscle there was considerable activity It was found to be moderately but significantly higher ($p<0\ 001$) in the middle part than in the proximal part whereas it was clearly lowest in the distal one The rate of oxidative deamination effected by the liver was about five times as high as that effected by the retractor penis But the deamination velocity accomplished by the kidney was up to twice as high as that effected by the liver

Discussion

Retractor penis muscle Active reuptake constitutes the major mechanism of elimination of released noradrenaline at various sympathetic nerve endings (Hertting *et al* 1961 Folkow *et al* 1968) A minor portion of the released transmitter may be O methylated by the COMT located in close proximity to the adrenergic receptor

TABLE II Monoamine oxidase activity in some organs of the bull expressed in μg of 5-HT metabolized/g of fresh tissue/hr (mean ± S D) Number of test animals in parentheses

Part of retractor penis muscle			Liver	Kidney
Proximal	Middle	Distal		
560±62 (7)	00+47 (7)	480±44 (7)	3300 700 (4	6000±1400 (4)

(Axelrod 1966) The low COMT activity observed in the bull retractor penis is in agreement with these conceptions There is no need for a rapid inactivation of released noradrenaline if the assumption is correct that this muscle is kept in an almost continuous contraction by impulses conducted along sympathetic nerves The mechanism of relaxation of the muscle at penile erection will be discussed in another connection (Klinge 1969b)

In the sympathetic nerves mitochondrial MAO inactivates the noradrenaline liberated from its more firmly bound store (Axelrod 1966) Histochemical studies of the distribution of MAO in several mammalian tissues (Koelle and de Valk Jr 1954) indicate that a minor part of this enzyme present in a tissue such as the bull retractor could be located in the unstriped fibres of the muscle itself as well as in those of the blood vessels although the main part should be found in the sympathetic nerves This does not however explain why the MAO activity was moderately but significantly higher in the middle than in the proximal part of the muscle The amount of catechol amines is about equal in both of these parts (Klinge 1969a) So far there is no evidence of the presence of 5 HT in any part of the muscle Due to its function and to anatomic conditions the muscle is probably most strongly *stretched in its middle part but the demonstration of a connection between this* situation and the higher MAO activity is lacking

Liver and kidney In several mammalian species the highest MAO activity is exhibited by the liver and kidney (Blaschko *et al* 1937, Bhagvat *et al* 1939, Tipton 1966) Which one of these organs shows a higher activity depends, among other things on the animal species and the substrate employed According to the present results the bull kidney shows a higher rate of oxidative deamination than the liver Using the manometric technique and tyramine as substrate Bhagvat *et al* (1939) arrived at the contrary conclusion

The COMT activity observed during the present study in the bull liver is in agreement with that measured in the liver of the cow by Axelrod and Tomchick (1958 According to these investigators there is wide variation in the COMT activity exhibited by the liver of different mammalian species This also seems to apply to the kidney since they found in the rat kidney a considerably higher COMT activity than that measured by the author in the bull kidney with the same technique The physiological significance of those species variations remains to be solved

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Left Ventricular Pressure and Maximum Rate of Pressure Rise as Determinants of Myocardial Oxygen Consumption during Hemorrhagic Hypotension in Dogs

By

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Abstract

Blegge-Asperheim, B and J Kjekshus *Left ventricular pressure and maximum rate of pressure rise as determinants of myocardial oxygen consumption during hemorrhagic hypotension in dogs* Acta physiol scand 1970 78 174—183

The influence of systolic left ventricular pressure (SLVP) and maximum rate of rise of left ventricular pressure (dP dt) on left myocardial oxygen consumption (MVO_2) has been studied in intact

d

infusion of angiotensin there was a marked elevation of SLVP but only small increases in MVO_2 and dP dt

On the basis of the changes in MVO_2 SLVP and dP dt it was shown that the contribution to an increase in MVO_2 of a rise in SLVP is accounted for by the concomitant increase in dP dt

A number of factors influence myocardial oxygen consumption (MVO_2), but their relative importance has not yet been clearly defined MVO_2 has been shown to vary with left ventricular systolic pressure (SLVP), ventricular dimensions and heart rate (Starling and Visscher 1927 Katz and Feinberg 1958 Sarnoff *et al* 1958 Rodbard *et al* 1964 McDonald *et al* 1966)

The maximum rate of rise of left ventricular pressure (dP dt) has been found to give a close relationship to MVO_2 in the controlled heart preparation (Sonnenblick *et al* 1965) Relationship has also been found between SLVP and dP dt (Reeves *et al* 1960, Gleason and Braunwald 1962) Administration of catecholamines which alter the inotropic state of the myocardium greatly increases MVO_2 and dP dt even without changing SLVP or tension (Krasnow *et al* 1964 Sarnoff *et al* 1965 Sonnenblick *et al* 1965)

The purpose of the present study was to investigate whether increased MVO_2 associated with increased SLVP might be correlated to and explained by the changes induced in dP/dt, so that SLVP would not have to be considered as an independent determinant of MVO_2

Such experiments at various pressure loads should preferably be performed without alterations in end diastolic volume, as a change in ventricular volume might in itself influence both myocardial oxygen utilization (Starling and Visscher 1927, Rodbard *et al* 1964, Rolett *et al* 1965) and dP/dt (Siegel and Sonnenblick 1964, Schaper *et al* 1965) Stable ventricular volumes could be achieved in hypovolemic dogs The left ventricular end diastolic volume decreased somewhat after bleeding, but was later relatively unaffected when the heart was subjected either to increased pressure load or to changing level of catecholamine stimulation SLVP and dP/dt were altered in the hypovolemic dogs by infusion of angiotensin and noradrenaline The infusions were again carried out after adrenergic α receptor blockade with phenoxybenzamine We could thus test the effect on MVO_2 of pressure loading alone, of the combined effect of pressure loading and inotropic intervention, and of inotropic intervention alone

Materials and Methods

13 mongrel dogs weighing between 15 5 and 23 kg were anesthetized by i v administration of pentobarbital 25 mg/kg b w, and additional doses of 1 mg/kg b w were given when necessary Sodium heparin, 3 mg/kg b w given i v was used as anticoagulant Trachea was intubated but ventilation was not assisted

When [illegible]

were performed In 9 dogs the tubing to the reservoir was clamped and $\overline{AP}$ was raised to 130 mm Hg by i v infusion of noradrenaline in doses of 0 5—2 µg/kg b w per min Determinations of MVO_2 were then made In 3 of the animals $\overline{AP}$ was lowered to the hypotensive level by unclamping the tubing from the dog to the reservoir during continuous infusion of noradrenaline

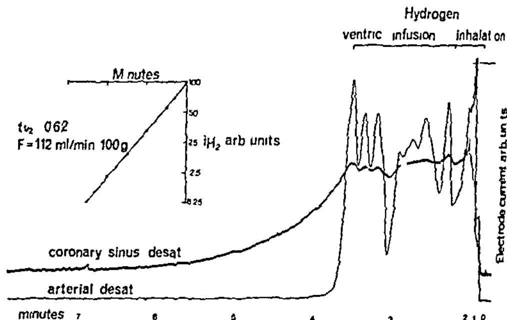
In a second series phenoxybenzamine 1 mg/kg b w was administered i v to 7 dogs The connecting tubing to the reservoir remained open in these dogs and spontaneous retransfusion occurred so that $\overline{AP}$ did not change After 30—45 min determinations of MVO_2 were repeated prior to and during i v noradrenaline infusion Doses of 1 5—2 0 µg/kg b w per minute did not result in changes in $\overline{AP}$ or in the blood volume of the reservoir 15 min after stopping the infusion no significant hemodynamic effect of noradrenaline remained and angiotensin was then given i v in 4 dogs in doses of 0 25 µg kg b w per minute with the tubing to the reservoir closed

$\overline{AP}$ [illegible]

Maximum rate of rise of LVP (dP dt) was determined with an RC differentiating circuit or by direct measurement of the maximum slope on the ventricular pressure curve In 3 animals, measurements of left ventricular pressure were also carried out with an intracardial micromanometer (Statham Physiological Pressure Transducer SF 1) There was close agreement between values obtained with the two pressure transducers

All investigations were performed at spontaneous heart rate

Cardiac output (CO) and left ventricular end-diastolic volume EDV) were measured by thermal dilution technique A copper constantan thermocouple was advanced into the ascending aorta from a femoral artery and the subsequent procedure was as previously described (Bugge Asperheim and Kjekshus 1968)



F g 1 Or g nal H_2 desaturation curves rec rde l s nultaneo sly n a rta and c ronary s nus Inset sem logari hm c plot of coronary s n us desaturat on curve w th est ma e l fl w (F) iH electric current

Myocard al bl ood flow MF was est mate l fr m l y lrogen gas desa urat on cur es (A kland *et al* 1967 H -concentratio d n
e l on the t p f a card ac
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t ssue was calculated fr m the half times of he mono-exponent al desa ura n c r es a c r t to the formula F (9 3 t , where t , s the half t me n m nu es H tissue bl od par n e ff c ent an l spec f c gra y was assu ed to be 1 00

Hydrogen gas was g en wi h the resp rat ry a r unt l stable concentra ns were rec rded in coronary s nus bl ood (as adm n stra n was then stoppe l and 30 50 ml f 0 9 % sal ne sa a ed w th hy lrogen at 1 atm was injecte l n the left ventr cle t ma nta a m an l d gen concentrat on n the m ocar l n f r an her 60 sec ns r g m ne f a e lesa ura n of a ma blood at the end f the nf s n H lr gen ox lat on current was meas re l w h Ke hley Instruments M cro It Am me er 153 A an l he utp t s gnal recorded a H neyw ll E own El c on ic Recorder

S multaneous desa ura n cur es fr aorta and c r nary s n s were t a ned n 2 d s and record ngs from ne of he l gs ar sh wn n F g 1 Desp te large and rap l a s ns arterial H_2-c ncen ra n dur ng n ra en r c lar nfus n the c ncen a n n the c ronary sinus rema ned fa rly s able n l ca ng g od equ l bra on of H n the m yoc r l w h maye den w h a less than 0 0 m n
They s all l ff red by less an

Arterial an l cor nary ven us l ood sa p s w a d s n l tan usl an l analyzed f r oxygen saturat on by the meth d f Aukland 19 Her ogl b n was as e l spec ph m metr cally as cyanmeth em oglob n Oxygen con ent was calcula e l by the n r s n fac r f 1 34 ml O g l emogl b n MVO was calcula ed as he prod c f c r nar bl od fl w and c r nary arter ovenous O_2 d fference

TABLE I Left ventricular hemodynamics and myocardial oxygen consumption after bleeding Effects of noradrenaline infusion with closed reservoir (9 dogs) and with open reservoir (3 dogs) Mean values ± S E

	Control	Hypovolemic hypotension 2 hours	Hypovolemia + noradrenaline	
			Closed reservoir	Open reservoir
SLVP	144	76	136	80
±S E	7	6	9	
dP/dt	3478	1790	5478	2166
±S E	433	254	965	
HR	159	166	181	166
±S E	12	10	11	
MVO_2	12 1	8 5	17 1	10 8
±S E	1 36	0 86	2 2	

SLVP systolic left ventricular pressure, mm Hg dP/dt maximal rate of rise of left ventricular pressure, mm Hg/sec HR heart rate MVO_2 myocardial oxygen consumption, ml/min 100 g

Results

Effects of bleeding

Pre-bleeding control MVO_2 varied considerably between dogs, with a range from 7 3 ml/min 100 g to 19 2 ml/min · 100 g (table I and II) This scatter of MVO_2 seemed to result from differences in inotropic stimulation, as there was good correlation between MVO_2 and dP/dt (r=0 93)

All animals showed a uniform response when bled from a mean aortic pressure of 147 mm Hg to 60 mm Hg The MVO_2 showed an initial decrease followed by a subsequent rise, with parallel changes in dP/dt and SLVP Stable values were reached after about 2 hrs when the experiments here reported were performed CO decreased from an average of 2450 ml/min to 960 ml/min, and remained essentially constant during the hypotensive period

EDV was followed in 2 dogs It was greatly reduced by the bleeding, from 44 5 and 60 ml to 20 ml and 18 ml, respectively Further changes in EDV during the hypotensive period, however, were small During noradrenaline infusion, EDV increased by 2 ml and 3 ml, and during angiotensin infusion by 8 ml and 2 ml

In all dogs, the large alterations in EDV induced by the bleeding were associated with only small changes in end-diastolic pressure (EDP) EDP either remained unchanged or decreased less than 2 mm Hg During noradrenaline and angiotensin infusions, slight increases in EDP (1—2 mm Hg) were observed in all dogs, suggesting similarity to the two dogs in which EDV was measured

TABLE II Left ventricular hemodynamics and myocardial oxygen consumption after bleeding Effects of phenoxybenzamine administration and of infusions of noradrenaline (dogs) and angiotensin (4 dogs) Mean values ± S E

	Control	Hypovolemic hypotension 2 hours	Hypovolemic hypotension +Phenoxy benzamine	Hypovolemic hypotension +Phenoxybenzamine	
				Noradrenaline	Angiotensin
SLVP	158	74	73	77	120
± S E	10	2	2	3	
dP dt	4257	2293	2479	4729	3475
± S E	520	361	371	352	
HR	173	193	205	216	182
± S E	17	17	14	17	
MVO_2	13 6	9 6	10 6	16 1	10 1
± S E	1 42	1 17	1 43	1 49	

SLVP systolic left ventricular pressure mm Hg dP/dt maximal rate of rise of left ventricular pressure mm Hg sec HR Heart rate MVO_2 myocardial oxygen consumption ml min 100 g

Effects of cardiac dynamics on MVO_2 during noradrenaline infusions

In experiments where the tubing to the blood reservoir was closed, noradrenaline markedly increased SLVP, dP/dt and MVO_2 Results from a typical experiment are shown in Fig 2 Heart rate increased slightly, and EDV was almost unchanged Similar results were obtained in 9 dogs (Table I) Mean increment in *SLVP during noradrenaline infusion was 60 (±7) mm Hg (SE of mean),* whereas the increase in dP dt was 3688 (±737) mm Hg per sec Heart rate changes were relatively small averaging 15 (±2) beats per min The combined effect of pressure and inotropic stimulation increased MVO_2 by an average of 8 6 (±1 7) ml min 100 g

Changes in per cent of prebleeding control are shown in Fig 3 upper left panel Under these experimental conditions MVO_2 SLVP and dP dt all increased During noradrenaline infusion CO increased from an average of 915 ± 94 ml min to 1220 ± 88 ml min Left ventricular external work CO SLVP increased considerably

In the three dogs where the tubing to the reservoir was unclamped so that AP and SLVP again stabilized on a hypotensive level during noradrenaline infusion small changes occurred *in dP dt and MVO_2 Fig 3 lower left panel, Table I)*

Effects of cardiac dynamics on MVO_2 during noradrenaline and angiotensin infusions after phenoxybenzamine administration

Phenoxybenzamine administration caused an uptake of 40—275 ml of blood from the reservoir CO increased from an average of 918 ± 101 ml min to 1643 ±

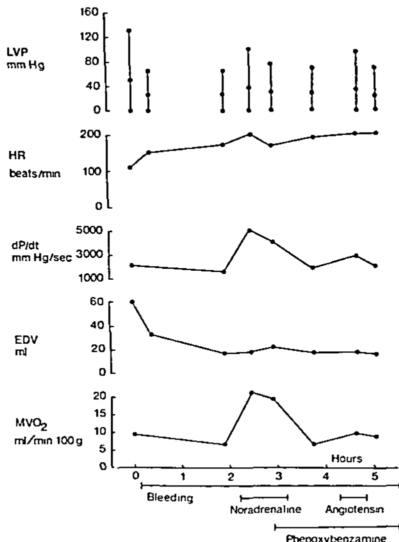


Fig 2 Effect of pressure load and inotropic state combined and separately on LV function and MVO_2. Blood pressure was lowered by bleeding to a reservoir before the experiment see Methods LVP = left ventricular pressure, HR = heart rate dP/dt = maximum rate of left ventricular pressure development, EDV = end diastolic volume, MVO_2 = left ventricular oxygen consumption

ml min Left ventricular external work consequently increased but was not associated with consistent changes in MVO_2 However, changes in dP/dt in individual experiments were followed by similar changes in MVO_2 (Table II) EDP did not change

After phenoxybenzamine administration, noradrenaline infusion had almost no effect on SLVP, and there was no bleeding into the reservoir A pure inotropic stimulation of the heart was thus obtained The increment in dP/dt averaged 2250±

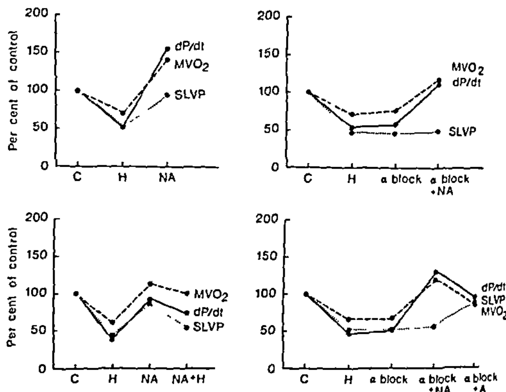


Fig 3 Effect of pressure loading and inotropic intervention on changes in MVO_2, SLVP and dP/dt. C = control (before start of bleeding), H = bleeding, NA = noradrenaline infusion, *a* block = phenoxybenzamine administration, A = angiotensin infusion.

412 mm Hg per sec MVO_2 increased nearly proportionally by an average of 5 5 ± 1 4 ml per min · 100 g Heart rate increased by 11 ± 7 beats per min Changes in per cent of prebleeding control are shown in Fig 3 (upper right panel), illustrating a close relationship between dP/dt and MVO_2, independent of the pressure load.

Angiotensin was then given in 4 dogs Fig 2 shows the results obtained in one experiment SLVP increased by an average of 49 mm Hg in these dogs — an increase slightly less than that obtained with noradrenaline infusion alone However, the increase in MVO_2, averaging 1 95 ml per min 100 g, was only 1/4 of that obtained with noradrenaline The increase in MVO_2 parallelled the changes in dP dt, averaging 1588 mm Hg per sec Changes in per cent of prebleeding control for all 4 dogs are shown in Fig 3 (lower right panel)

Discussion

Since the oxygen cost of the ejection period and the subsequent relaxation of the myocardium is low (Monroe 1964), it is reasonable to relate myocardial oxygen consumption (MVO_2) to variables of the isovolumic period, such as peak developed tension (McDonald *et al* 1966) and the rate of rise of left ventricular pressure (dP dt

(Sonnenblick *et al* 1965) No previous evaluation of the relative importance of these variables as determinants of MVO_2 has been undertaken in the intact dog

The present study, performed in hypovolemic dogs, indicates that systolic left ventricular pressure (SLVP) is not a primary determinant of MVO_2, as the correlation between SLVP and MVO_2 may be accounted for by the associated changes in dP/dt However, this statement requires some qualification, discussed in the following

Several investigators have demonstrated the importance of myocardial tension and closely related factors as determinants of MVO_2 Sarnoff *et al* (1958) found high correlation between MVO_2 and tension time index — the integral of the systolic portion of the aortic pressure curve It can be shown that SLVP as measured in the present studies is closely correlated to the tension time index (Aukland *et al* 1967) McDonald *et al* (1966) took the geometric factor into account and demonstrated that MVO_2 is better correlated with peak developed tension than with the tension time index Peak developed tension and SLVP would be equal as determinants of MVO_2 as long as end diastolic volume remains unchanged Small increments in ventricular volume were observed during both angiotensin and noradrenaline infusion in the present study The changes in peak developed tension might therefore be larger than the observed changes in SLVP However, minor changes in ventricular volume are of small consequence, as tension is proportional to the third root of ventricular volume

An increase in SLVP was associated with augmentation of dP/dt, independent of inotropic interventions, in our study as well as in several others (Reeves *et al* 1960, Zeig *et al* 1961, Gleason and Braunwald 1962, Wallace *et al* 1963) If dP/dt was the common determinant of MVO_2, both when MVO_2 was raised by increasing cardiac contractility and by raising aortic pressure, it would be expected that the ratio between changes in MVO_2 and dP/dt, $\Delta MVO_2/\Delta dP/dt$, might remain constant under all experimental conditions Even in these experiments when ventricular EDV showed small changes, this was not the case The ratio was larger during noradrenaline infusion than during angiotensin infusion For a similar rise in dP/dt, the elevation of MVO_2 during noradrenalin infusion was almost twice as high as during angiotensin infusion Further the ratio was greater during noradrenaline infusion in the phenoxybenzamine treated animals where the blood pressure remained unchanged than during angiotensin infusion These findings indicate that the increase in pressure during angiotensin infusion cannot be considered as an independent determinant of MVO_2 It might be argued that MVO_2 is less increased during angiotensin infusion on account of specific effects of angiotensin and that experiments performed under angiotensin infusion are therefore not representative of increased pressure load In other studies this objection has been rejected, as similar changes in dP/dt and MVO_2 were obtained by raising aortic pressure either by angiotensin infusion or by mechanical constriction of the descending aorta (Bugge Asperheim and Kiil 1969, Mjos and Kjekshus to be published)

The finding that an increase in dP/dt by raising ventricular tension at constant

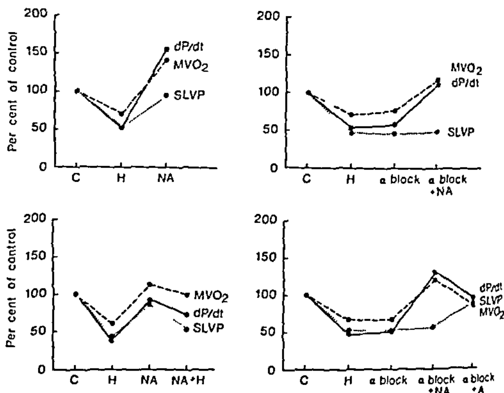


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412 mm Hg per sec. MVO_2 increased nearly proportionally by an average of 5.5 ± 1.4 ml per min. 100 g. Heart rate increased by 11 ± 7 beats per min. Changes in per cent of prebleeding control are shown in Fig. 3 (upper right panel), illustrating a close relationship between dP/dt and MVO_2, independent of the pressure load.

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The finding *that* an increase in dP/dt by raising ventricular tension at constant

inotropic stimulation has little effect on MVO_2 is in agreement with earlier studies of Graham *et al* (1966), but more recently (1968), Graham *et al* modified their views and suggest that both augmentation of contractility by catecholamine administration and increasing ventricular tension contribute equally to the increased MVO_2

The idea of dP/dt as a determinant of MVO_2 was introduced because neither tension nor SLVP could account for the increase in MVO_2 during catecholamine infusion The present studies show that SLVP is no independent determinant but they also suggest that the changes in MVO_2 during noradrenaline infusion are not accounted for by the increase in dP/dt It is not clear why an increase in dP/dt induced by catecholamine infusion affects MVO_2 more than when dP/dt is raised by increasing aortic pressure *The most likely possibility for an increase in* $\Delta MVO_2/\Delta dP/dt$ during noradrenaline infusion would be that catecholamines have metabolic effects on the heart that would not be expressed in changes in dP/dt It has been suggested several times previously that the oxygen-wasting effect of noradrenaline was due to an increase in the resting metabolism of the heart This view is supported by the recent finding of an increase in MVO_2 by raising the concentration of catecholamines in the perfusate of the isolated non-beating rat heart (Hauge and Øye 1966) The increase in MVO_2 of the non beating heart is small, however, compared with that induced by catecholamines in the intact organism, and it is therefore unlikely that an increase in resting metabolism is of great importance for the intact heart

Another metabolic effect requires more serious consideration Catecholamine infusion results in a large increase in plasma concentration of free fatty acids (FFA) and the myocardial uptake of FFA is also greatly increased Challoner (1968) has suggested that uncoupling of oxidative phosphorylation is increased by high FFA level during catecholamine infusion A possible interpretation is therefore that a catecholamine-induced increase in dP/dt is associated with a large increase in MVO_2 because high FFA reduces the metabolic efficiency of the heart

Prolonged hypotension by bleeding is known to stimulate the release of catecholamines (Walton *et al* 1959) and the present experiments have therefore probably been performed with a background of elevated arterial catecholamine concentrations *It should also be noted that only pressures below normal control pressures have* been examined resulting in almost unchanged ventricular end diastolic volumes both during increased catecholamine stimulation and during pressure loading For these and other reasons it is possible that different relationships may exist between MVO_2 and dP/dt at higher blood pressure levels, and that ventricular tension may then appear as a more important determinant of MVO_2

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Reflex Changes in Sympathetic Activity and Arterial Blood Pressure Evoked by Afferent Stimulation of the Renal Nerve

By

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Abstract

Aars H and S Akre. *Reflex changes in sympathetic activity and arterial blood pressure evoked by afferent stimulation of the renal nerve* Acta physiol scand. 1970 *78* 184—188

The effects of afferent stimulation of renal nerves on efferent sympathetic activity and systemic blood pressure were studied in anesthetized rabbits In most animals afferent impulses produced reduction or abolition of sympathetic activity and a fall in blood pressure High frequencies of stimulation (20—30 pulses sec or more) caused a more marked and long lasting depression of efferent activity and blood pressure than low frequencies The highest sensitivity of blood pressure to variations in stimulation frequency was found in the range of 1—10 pulses sec Stimulation of the renal afferents had qualitatively the same effect on sympathetic activity and blood pressure as aortic nerve stimulation but for the aortic nerve higher stimulation frequencies were required to obtain the same reduction in blood pressure It is concluded that afferent activity in renal nerves has a predominantly inhibitory effect on vasomotor activity resulting in a fall in blood pressure

In addition to their efferent fibres the renal nerves are known to carry afferent fibres which are activated by elevation of the intrarenal pressure (Niijima 196[illegible] Åström and Crafoord 1967 1968 Ueda Uchida and Kamisaka 1967) The functional significance of this activity remains uncertain. Whereas Ueda *et al* (1967) showed that afferent activity in the renal nerve elicited a reflex depression of efferent sympathetic activity and a fall in systemic blood pressure Åström and Crafoord (196[illegible]) found that afferent renal nerve activity had no effect on blood pressure

The present work is a study of the effects of electrical stimulation of afferent fibres in the renal nerve on efferent sympathetic activity and arterial blood pressure in rabbits The responses were compared to the effects of stimulation of the aortic nerve

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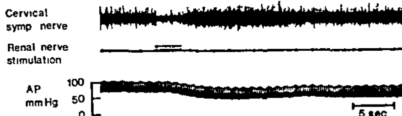


Fig 1 Effect on activity in left cervical sympathetic nerve and systemic blood pressure by afferent stimulation of the renal nerve (20 pulses/sec 1 msec 4 V) in 3 sec Stimulation marked by signal Systemic blood pressure (AP) recorded from right brachial artery

Material and Methods

Experiments were performed on 21 adult rabbits anesthetized with 3 ml 1 % chloralose/kg and 3 ml 25 % urethane/kg One half of the total amount was given i v the rest i p Urethane was supplemented during the experiments when necessary Rectal temperature was kept between 36 and 37 5° C, using a heating lamp and covers Brachial or femoral artery pressures were measured with a Statham transducer The animals were tracheotomized to secure free airways

The sympathetic and aortic nerves on both sides of the neck were exposed but not cut Left renal nerves were reached through a lateral incision in the abdominal wall Two nerve bundles, usually one on each side of the renal artery were dissected free in a length of about 10 mm Both nerves were cut distally One nerve was used for afferent stimulation the other for recording efferent activity Bipolar platinum electrodes were used for both purposes and for stimulation of the left aortic nerve Nerve strands used for stimulation and recording were bathed in liquid paraffin to prevent drying The renal nerve was stimulated with square wave pulses of 0 5—30 V amplitude and 1—5 msec duration delivered from a Grass stimulator via an isolation unit The frequencies ranged from 1—60 pulses/sec The aortic nerves were stimulated with pulses of 6—12 V 0 1—1 msec 10—120 pulses/sec Stimulation periods were 3 or 20 sec

The neurograms and the arterial blood pressure were recorded on a jet ink writer (Elema Mingograph) In some experiments the arterial blood pressure and integrated sympathetic nerve activity (Aars and Akre 1968 were recorded on a Sanborn recorder

Results

Stimulation of the proximal end of the cut renal nerve caused reduction or cessation of efferent sympathetic activity and a fall in systemic blood pressure This is shown for two animals in Fig 1 and 2 with neurograms from cervical and renal sympathetic nerves respectively *The reduction in efferent renal sympathetic activity was* found to appear 180—230 msec (average 200 msec) after start of stimulation and the fall in blood pressure followed 2—3 sec later The responses to afferent stimulation of the renal nerve were similar to those of aortic nerve stimulation as shown in Fig 2

The inhibition of efferent sympathetic activity increased with increasing frequencies of afferent renal nerve stimulation (Fig 2) as did the reduction in blood pressure In Fig 3 maximum fall in diastolic blood pressure is plotted against the respective frequencies used for stimulation The blood pressure response to afferent stimulation of the renal nerve was equal at 4 V and 12 V For the aortic nerve at

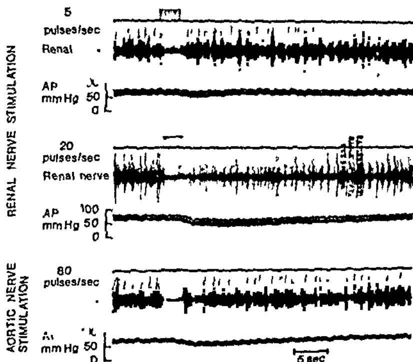


Fig 2 Effect on efferent renal nerve activity and systemic blood pressure by stimulation of renal afferents with 5 and 20 pulses/sec (1 msec 12 V) and by stimulation of left aortic nerve with 80 pulses/sec (0 1 msec 6 V) Stimulation periods of 3 sec marked by signal

near optimal intensity more than four times higher stimulation frequencies were required to obtain the same fall in blood pressure as with stimulation of renal afferents The blood pressure response was most sensitive to variations in stimulation frequencies in the low frequency range which can be seen from the initial slope of the response curves The greatest fall in blood pressure was obtained with 20–30 pulses/sec for renal afferents and 100–120 pulses/sec for the aortic nerve In some animals the reflex response to renal nerve stimulation diminished when higher stimulation frequencies were used

Increasing the intensity of the electrical pulses during afferent stimulation of the renal nerve — in order to evoke activity in the thin C fibres did not result in a reversal of the responses (Fig 4 A similar change of stimulation characteristics has been found to reverse a depressor into a pressor response in afferent stimulation of mixed somatic and splanchnic nerves (for references see Johansson 1962 Johansson and Langston 1964 Although 40 times the threshold voltage and up to 60 pulses/sec were utilized no reversal of the blood pressure response was observed in our experiments However when stimulation periods of 20 sec were used the efferent activity returned during the stimulation and usually earlier and more fully with high frequency stimulations Occasionally the efferent renal nerve activity exceeded the control level in the last part of the stimulation period This was in

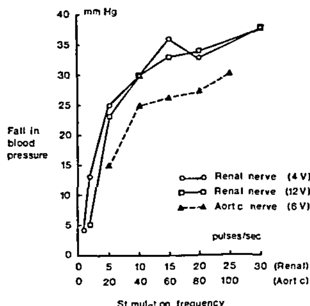


Fig 3 Afferent stimulation of aortic and renal nerves in 3 sec periods in one animal Relationship between stimulation frequency and fall in diastolic blood pressure Blood pressure returned to control level between stimulation periods

contrast to aortic nerve stimulation which always caused depression of efferent sympathetic activity and a fall in systemic blood pressure

In three experiments an inconsistent pressure rise was seen during stimulation of renal afferent fibres This pressure response could be induced by stimulation with pulses of 1—10 V and frequencies from 10—60 pulses/sec The blood pressure responses to afferent renal nerve stimulation were not qualitatively altered by barodenervation assisted positive pressure respiration or administration of a muscle relaxant (Flaxedil 2 mg/kg)

Discussion

In the present experiments on rabbits in chloralose urethane anesthesia afferent stimulation of the renal nerve caused a reflex reduction or abolition of efferent sympathetic activity and a fall in systemic blood pressure This is in accordance with the findings of Ueda *et al* (1967) that increased afferent activity in the renal nerves

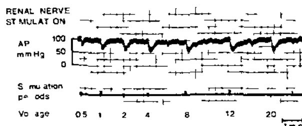


Fig 4 Effect on systemic blood pressure by stimulating renal afferents (10 pulses/sec 1 msec with increasing voltages Stimulation periods of 3 sec indicated by signal

— produced by elevation of the intrarenal pressure — led to a reduction in efferent renal nerve activity and a fall in blood pressure in the dog Our results are in contrast to those of Åstrom and Crafoord (1968), who in cats and rats found no blood pressure responses to procedures which increased activity in the afferent fibres of the renal nerves The reasons for the discrepancy are unknown

The responses elicited by stimulation of renal afferents were similar to the effects of stimulation of a baroreceptor nerve, both with respect to the response pattern in the neurogram and the blood pressure deflections Definitely higher stimulation frequencies were required to obtain similar depressor effects with stimulation of the aortic than the renal nerve, and the maximum depressor effects of afferent stimulation of renal nerves usually surpassed the maximum depressor response obtained by aortic nerve stimulation Vasomotor neurons were thus more affected by afferent impulses from the kidney than from the aortic baroreceptors It should also be noted that the relationship between reduction in renal efferent activity and the fall in systemic blood pressure was the same in stimulation of renal afferents and the aortic nerve This is contrary to the observations that changes in baroreceptor activity mainly affected the vascular bed in skeletal muscle and left the renal vessels almost unaffected (Lofving 1961) In the present experiments, changes in efferent activity in the renal nerve always reflected total sympathetic activity — as judged from the corresponding changes in blood pressure

The lack of a pressor response when the characteristics of the stimulation were changed to include stimulation of C fibres suggests the existence of only a few C-fibres in the strands of renal nerve employed or that the excitatory effects of C fibre activity were masked by the stronger depressor effects of activity in other fibres The similarity between the results of electrical stimulation and of increasing the activity by more natural means (Ueda Uchida and Kamisaka 1967) points to a dominatingly inhibitory sympathetic effect of afferent activity in the renal nerve

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Effects of Hemorrhagic Shock and Treatment with Hypothermia on the Potassium Content and Transport of Single Mammalian Skeletal Muscle Cells

By

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Abstract

HALJAMÄE, H *Effects of hemorrhagic shock and treatment with hypothermia on the potassium content and transport of single mammalian skeletal muscle cells* Acta physiol scand 1970 *78* 189—200

The effect of in vivo hemorrhagic shock and its treatment with hypothermia was studied by following the temporal pattern of intracellular potassium content in single skeletal muscle fibres during in vitro incubation For the control cells isolated before the different experimental procedures were instituted, reproducible changes in potassium content were observed during the period of incubation The potassium content of cells isolated shortly after the taking of the biopsy was somewhat lower than of cells isolated after 10 min of incubation After 10 to 15 min of incubation a marked loss of cellular potassium was seen, followed by a period of active reaccumulation After hemorrhagic shock under normothermal conditions the potassium content of the cells was markedly lower and no active cellular potassium reaccumulation was obtained during prolonged incubation Hemorrhagic shock treated with hypothermia lowered the initial potassium content somewhat but both the periods of cellular potassium loss and active reaccumulation were seen Hypothermia alone only slightly modified potassium transport during the first 10 to 15 min of in vitro incubation

The results are discussed from the standpoint of cellular metabolism and high-energy phosphate production with special reference to the exchange of substances between body compartments under the diffrent experimental conditions

The effect of hemorrhagic shock on the electrolyte composition of skeletal muscle is still a matter of dispute No significant change (*e g* Marcynska *et al* 1966) or an increase (*e g* Brand 1966) in the potassium content of muscular tissue biopsies has been reported From analyses of single skeletal muscle cells a decrease averaging 26 % of the cellular potassium content, has been shown to occur after shock in dogs (Hagberg, Haljamae and Rockert 1967) Results indicating a decrease of cellular potassium concentration were obtained by Shires *et al* (see Shires and Carrico 1966) who employed a modified Ling electrode to demonstrate a marked reduction in transmembrane potential of rat skeletal muscle fibres after shock Hagberg Haljamae and Röckert (1968) sampled nanoliter quantities of local tissue fluid before and

after hemorrhagic shock in dogs and found a much greater increase of the potassium content of tissue fluid than of blood plasma indicating a local interstitial electrolyte accumulation

The results of experiments at the cellular level thus imply an impairment of the metabolic activity of skeletal muscle during the hypoxia of shock Impaired tissue energy production during shock has been shown by LePage (1946a, 1946b) Grigor'eva, Radzievskii and Shchukina (1965) found a marked reduction of ATP and CrP levels in rabbit skeletal muscle after occlusion of the femoral artery

In a previous report (Haljamae 1969) methods for the isolation of single skeletal muscle cells during in vitro incubation of a muscle biopsy and the analysis of their electrolyte content by X-ray fluorescence microanalysis or ultra-micro flame photometry were described It was also shown that marked changes in the potassium content of these cells took place during the first 30 to 40 min of incubation These changes were shown to be dependent on available cellular energy In the present investigations these analytic methods have been used to study the response of skeletal muscle cells to the anoxic injury and metabolic impairment caused by shock Shock treatment with hypothermia was also tried to see if the cellular response to shock could be modified by decreasing the metabolic rate of the tissues during the period of hypoxia caused by shock

Methods

Dogs of mixed breed and both sexes, weighing 10—20 kg were used Anesthesia was induced with Mebumal (pentobarbital) sodium (30—40 mg/kg) and the dogs were heparinized (2.5 mg/kg) A plastic cannula was then inserted into one of the carotid arteries and connected to a y tube One limb of the y tube was attached to a pressure transducer and recorder and the other limb led to a bleeding cup A tracheostomy was performed and the dogs were artificially respirated with air A muscle biopsy was taken from the adductor muscle of one of the hind legs Single skeletal muscle cells were dissected out in an oxygenated (O_2 95 %, CO_2 5 %) Krebs solution maintained at pH 7.3 (bicarbonate buffered) and 20—22° C according to the methods previously described (Haljamae 1967 1969) Three groups of dogs were now treated differently

Group A Hemorrhagic shock was induced by bleeding until the blood pressure in the carotid artery was 45 mm Hg The dogs were maintained at this blood pressure for 135 min At the end of this time a new muscle biopsy was taken from the adductor muscle of the contralateral hind leg and single skeletal muscle cells were isolated during the in vitro incubation No respiration support was given to these dogs

Group B Hemorrhagic shock was induced as above The dogs were artificially respirated with air As soon as a constant arterial blood pressure of 45 mm Hg was obtained the dogs were transferred to a box for controlled surface cooling Thermocouples were inserted into the rectum oesophagus into a muscle of the hind leg and beneath the skin Cold air cooling was continued until the oesophageal temperature was 30 C at which time the dogs were removed from the cooling box After the dogs were moved back into 20—22 C surroundings the [illegible] oesophageal temperature continued to decrease to about 28 C before rising again After the same time period (135 min) of shock as received by the dogs in group A a new muscle biopsy was taken and single skeletal muscle cells isolated

Group C The dogs in this group were not bled but only subjected to the same degree and [illegible]

[illegible] were [illegible]

transferred to a clean mylar foil The surplus dissection solution was [illegible] with a thin pointed pipette and the cells dried within a few seconds The dissection was continued for 40—50 min during which time period 10—20 single cells were isolated The [illegible] period in the dissection solution i e the time between the taking of the biopsy and the isolation

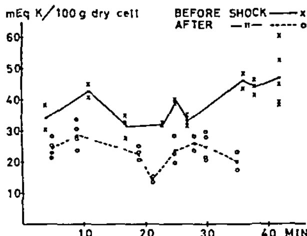


Fig 1 Effect of hemorrhagic shock on the temporal changes of the potassium content in single skeletal muscle cells during in vitro incubation as obtained from ultra micro flame photometric analysis

of each cell on the mylar foil was carefully noted Phase contrast microscopic examination of the cells was performed to ascertain the extent of cellular injury caused during the isolation procedures From the central part of cells fulfilling the criteria for uninjured cells' (see Haljamae 1967, 1969) 3—5 pieces 70—90 μ long were cut out and separately analyzed There fore several electrolyte concentration values could be obtained for each cell The dry mass of each cut out piece was obtained from x ray absorption measurements by the method of Rosen gren (1959) Potassium content was determined by two different micro-methods x ray fluorescence microanalysis (Long and Rockert 1963) and ultra micro flame photometry (Halja mae and Larsson 1968) The application of these two analytic micromethods for analysis of single skeletal muscle cells has been described in previous reports (Haljamae 1967 1969) X ray fluorescence microanalysis was performed directly on the dry cut out pieces However, a 2 hr extraction with 70 8 nanoliters of quartz redistilled water or 2 N nitric acid under liquid paraffin cover was required before duplicate or triplicate analyses of 21 8 nanoliter samples of the extract could be performed with the ultra micro flame photometer

Results

Group A dogs Hemorrhagic shock

Significant differences in intracellular potassium content were found between skeletal muscle cells isolated before and after the induction of hemorrhagic shock The time courses of potassium content changes in these two groups of cells also varied markedly during in vitro incubation

Fig 1 illustrates temporal changes of potassium content as obtained from ultra micro flame photometric analysis of cells isolated from one experimental animal before and after shock For every cell the value for each different cut out and separately analyzed piece is graphed The curves display the calculated potassium content of each cell *i e* the mean value of the separately analyzed pieces as a func tion of the time of in vitro incubation As can be seen from Fig 1 the potassium content of all the cells isolated before shock was higher than that of cells isolated after shock Before shock there were also changes in the cellular potassium content with time Cells isolated shortly after taking the biopsy had a lower potassium con

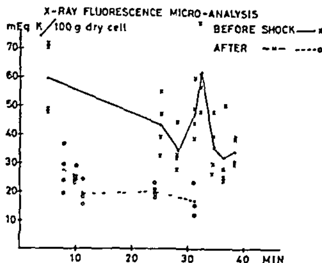


Fig 2 X ray fluorescence micro analytic results of the effect of *hemorrhagic shock on the potassium content in muscle cells during in vitro incubation*

tent than cells isolated a few minutes later Cells isolated 15—25 min after taking of biopsy had a much lower potassium content than cells isolated after the period of initial regain or after 30—50 min in the incubation medium Following the period of cellular loss of potassium a reaccumulation always took place (*cf* Haljamäe 1969) After shock no reaccumulation of intracellular potassium to pre shock values could be demonstrated

The potassium content of cells isolated before and after shock was also determined by x ray fluorescence analysis Sample results are shown in Fig 2 Variation in the potassium values obtained for the different cut out pieces from each cell was greater with this method than with ultra micro flame photometry With this analytic method also the potassium content of cells isolated after shock was markedly lower than that of cells isolated before shock For cells isolated before shock a tendency for cellular potassium loss followed by *reaccumulation* was seen but not for cells isolated after shock

Fig 3 represents the averaged potassium content with time of in vitro incubation of single skeletal muscle cells taken from 4 dogs 32 different cells (195 cut out pieces) were analyzed before hemorrhagic shock and 28 cells (93 cut out pieces) after The curves display the average value of all cells isolated within 5 min intervals of incubation As can be seen from the figure the above mentioned potassium shifts with time *i e* loss of intracellular potassium after about 10 min of in vitro incubation followed by cellular potassium reaccumulation could be clearly demonstrated for the cells taken before shock After the hemorrhagic shock the cellular potassium content was considerably lower and no reaccumulation of intracellular potassium was obtained The average pre hemorrhagic potassium content of cells isolated after 10—15 min of incubation was 16 9 mEq/100 g dry muscle cell and was significantly higher ($P < 0.01$) than the corresponding posthemorrhagic value of

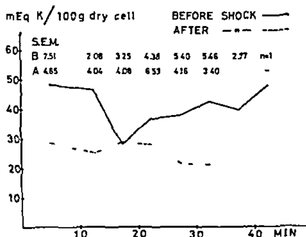


Fig 3 Averaged potassium content changes of cells isolated within 5 min intervals during in vitro incubation before and after hemorrhagic shock

25.5 mEq/100 g There was also a significant difference ($P<0.02$) between pre and posthemorrhagic values after 30—35 min of incubation *i e* after the reaccumulation of potassium in the prehemorrhagic cells had taken place

Group B dogs Hemorrhagic shock + hypothermia

Fig 4 is a sample record of the potassium content of cells taken before and after hemorrhagic shock treated with hypothermia and analyzed by micro flame photometry

The values for each cut out and separately analyzed piece from each cell are given in the graph Fig 5 displays potassium content averaged over 5 min intervals against incubation time for cells from 6 experimental animals subjected to shock and hypothermia All together 69 cells (160 separate cut out pieces) were analyzed before hemorrhage + hypothermia and 57 cells (139 pieces) after

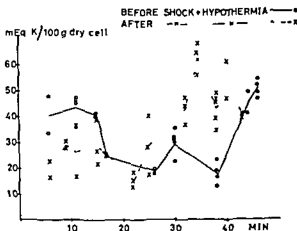


Fig 4 Cellular potassium content and transport characteristics of cells from one animal subjected to hemorrhagic shock followed by treatment with hypothermia

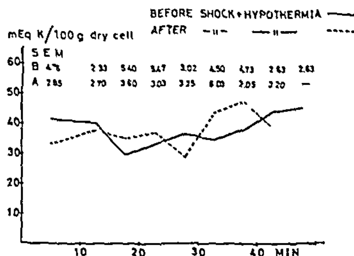


Fig. 5. Averaged 5-min interval values of the potassium content of cells during in vitro incubation before and after shock treated with hypothermia in 6 dogs. S.E.M. values tabulated.

As can be seen from Fig. 4 and 5 the previously observed potassium changes with incubation time could de demonstrated in the control cells taken before shock + hypothermia. The potassium content in cells isolated within the first 10 to 15 min of incubation was lower after shock + hypothermia than in control cells. However in contrast to the effect of hemorrhagic shock only a complete reaccumulation of intracellular potassium to values characteristic of the control cells was observed after incubation times of 30 to 40 min.

Differences between cells from different animals in the temporal pattern of cellular potassium loss and reaccumulation increases the variability about the mean

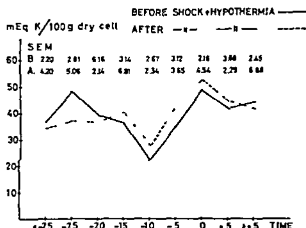


Fig 6 Effect of shock treated with hypothermia on the cellular potassium content and transport when the value for completed potassium reaccumulation in each experiment is taken as a fixed time (zero-time value) and cells isolated within successive 5 min periods before and after this value are grouped and averaged from the different experiments

Group C dogs Hypothermia

The averaged curves of the potassium content of cells from 4 dogs subjected to hypothermia only are shown in Fig 7

38 cells (85 separate cut out pieces) were analyzed before hypothermia and 39 cells (95 pieces) after The values are averaged and graphed as in Fig 6 The same pattern of electrolyte shifts in relation to incubation time as seen previously in other control animals was observed in this case After hypothermia a period of net potassium gain during the first 5—10 min of incubation was absent The difference between the potassium content of experimental and control cells within this initial incubation period was, however, not significant ($P<0.2$ for cells isolated 20—25 min before completed reaccumulation, $P<0.6$ for cells isolated 15—20 min before reaccumulation) Reaccumulation of intracellular potassium to concentrations equivalent to those of the control cells was obtained after the in vivo hypothermia

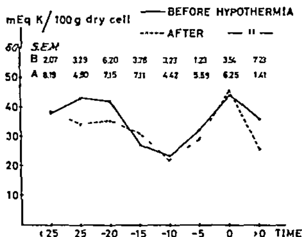


Fig 7 Potassium content of cells during in vitro incubation before and after induced hypothermia Results from 4 dogs are grouped as in Fig 6 using the value for completed potassium reaccumulation as zero-time value

Discussion

Effect of hemorrhagic shock

The potassium content of skeletal muscle cells after hemorrhagic shock was markedly lower than that of control cells indicating an in vivo loss of potassium (*cf* Hagberg Haljamäe and Rockert 1967) No such marked difference is found if macrosamples of muscular tissue are analyzed before and after shock (Marcynska *et al* 1966 Brand 1966, Johnson Jr and Tucker Jr 1968) The discrepancy between the results obtained from analysis of macrosamples of tissues and those from single cell analysis may be attributable to a local accumulation of potassium in the interstitial fluid (Hagberg Haljamae and Rockert 1968), which can not be corrected for in the analysis of macrosamples Day (1952) showed that connective tissue profoundly limits free diffusion due to its mucopolysaccharide content, since hyaluronidase treatment produced a 10 fold increase in saline penetration Similar restriction in the diffusion of dyes through the interstitial space of frog mesentery were reported by Wiederhielm (1966)

During shock the blod flow to the hind leg of dogs is very low (Bond Manley and Green 1967) and the blood flow in the capillary bed of the tissues may be further restricted by arterio venous shunting of blood (see Gourzis and Nickerson 19… and the presence of a slowly circulating red cell volume (Shoemaker 1962) The initial stage of shock is also characterized by a net inward movement of extravascular fluid to the intravascular compartment (Mellander and Lewis 1963) This movement must involve a partial dehydration of the interstitial fluid spaces further limiting electrolyte exchange through the tissue fluid spaces The markedly decreased lymph flow during shock also indicates a diminished interstitial fluid circulation (Wessely 1958) The several effects listed above promote local accumulation of metabolites and electrolytes from the hypoxic cellular tissue

The loss of plasma from the intravascular compartment seen during later stages of shock may be due to a deleterious effect of accumulated metabolites on precapillary resistance (Mellander and Lewis 1963) but an increased interstitial osmotic pressure due to accumulated electrolytes and metabolites may contribute A localized accumulation of potassium as such will probably also promote a reduction in vascular resistance (Skinner Jr and Powell Jr 1967) It is thus significative that it is not until the later part of hypotensive shock characterized by increased hydration of the interstitial tissue that a more marked increase in plasma potassium takes place (Bowers *et al* 1945 Danoff and Greene (1964 induced vasodilatation with hexamethonium after a period of moderate hypovolaemic shock (depletion of 25 % of estimated blood volume in dogs and found that if the ganglionic blocking agent was given a higher value for excess lactate in blood than for untreated hypovolemic dogs was demonstrable As suggested by the authors this increase could be due to an increase of the capacity of the vascular system with concomitant decrease in blood volume and thus further decrease in tissue oxygenation as venous return and cardiac output will be reduced It could however also partly be due to an increased

hydration with a higher wash out rate of locally accumulated metabolites When considered on the basis of circulatory changes and tissue hydration changes during shock the lower initial potassium content of cells after shock found in the present investigation would well agree with an in vivo local interstitial accumulation not revealable from analyses of plasma or macrosamples of tissue

During hemorrhagic shock an oxygen deficit develops rapidly (Crowell and Smith 1964) There is an increase in inorganic phosphorus and in the lactate/pyruvate ratio in blood coupled with a decrease in blood glucose levels and tissue glycogen content indicating a predominance for anaerobic glycolysis (see Shoemaker 1967) In the anaerobic tissues the lactic acid production is higher and less of the glucose metabolism is directed towards the pyruvic acid—acetyl coenzyme A step and less pyruvic acid is transformed into oxaloacetic acid (see Schumer and Kukral 1968) Since anaerobic metabolism yields less ATP the production of energy rich phosphate compounds is impaired The cellular storage supply of energy in the form of creatine phosphate is depleted during impaired tissue perfusion (*cf* Grigor'eva Radzievskii and Shchukina 1965) The avialable cellular energy may therefore be too limited to keep up with the needs of the sodium pump resulting in cellular loss of potassium and gain of sodium Therefore it is not surprising that the initial potassium content of cells isolated after the shock period is low Since the high-energy phosphate stores are markedly depleated (LePage 1946a 1946b) no active potassium gain takes place during prolonged incubation in the shock cells There is apparently some energy production during the incubation of the shock cells since the potassium content does not very rapidly decrease under the in vitro conditions High cellular potassium content and the ability of the cells to reaccumulate potassium (during in vitro incubation) is dependent on an intact ion pumping system and energy production (Haljamäe 1969) Metabolic inhibitors interfering with the efficiency of the sodium pump (ouabain) or high-energy phosphate production (monoiodoacetate 2 4-dinitrophenol) were added to the incubation medium producing a loss of cellular potassium content and preventing potassium reaccumulation during prolonged incubation Therefore the changes in cellular potassium content and transport observed after hemorrhagic shock are in agreement with a marked depletion of cellular energy

Effect of treatment with hypothermia

The effect of hypothermia in the treatment of shock is still controversial Many reports have claimed a markedly increased survival rate (*e g* Blalock and Mason 1941 Postel Reid and Hinton 1957) while others indicate a deleterious effect (Wilson *et al* 1956)

Hypothermia in normovolemic dogs does not seem to cause marked effects on intermediary metabolism Drucker Kingsbury and Graham 1962 did not find any accumulation of inorganic phosphorus or excess lactate in hearts of these animals The degree of hypothermia used in the present experiments body temperature down to 28 C will decrease the metabolic rate to about 40 60 % of normal

(Brewin 1964) The effect of in vivo hypothermia on the electrolyte content in single skeletal muscle cells during in vitro incubation seems small The period of potassium reaccumulation during the first 10 min of incubation was evident before but not after in vivo hypothermia This difference might be due to the lower metabolic rate of cells taken after hypothermia The periods of cellular potassium loss and reaccumulation seen during prolonged incubation are similar to those found in control cells

After shock treated with hypothermia the cells isolated early in the incubation period had a lower potassium content than control cells indicating an in vivo loss. *This difference is on the average 8 6 meq/100 g for cells isolated during the first* 10 min of incubation The corresponding average difference between control cells and cells taken from normothermal shocked animals is 19 7 meq/100 g It has also been shown that in shock animals treated with hypothermia there is a moderate increase (average 2 0 meq/l) in the potassium content of local tissue fluid but only a slight increase (average 0 3 meq/l) in plasma potassium concentration (Hagberg Haljamae and Rockert 1969) The corresponding changes were much higher for normothermal animals in shock (increase of tissue fluid K, 5 5 meq/l, plasma K 2.0 meq/l Hagberg Haljamae and Rockert 1968) Therefore the results from analysis of cellular potassium content as well as those of tissue fluid and plasma potassium indicate that the cellular injury during hemorrhagic shock is markedly reduced after treatment with hypothermia

The potassium changes during prolonged in vitro incubation in the cells from hypothermically treated animals also demonstrate that the cellular metabolism is not as badly impaired as after hemorrhagic shock only The initial period of regain of intracellular potassium seen in control cells is absent in shocked + hypothermically treated cells However the later periods of cellular potassium loss and reaccumulation are clearly demonstrable Therefore it can be assumed that the cellular metabolism is less impaired since the reaccumulation of intracellular potassium during in vitro incubation is energy dependent (*cf* Haljamae 1969

Artificial respiration was used in the present investigation to prevent the combined effects of the mainly metabolic acidosis of shock and the respiratory acidosis of hypothermia The artificial respiration given to the dogs in shock which were treated with hypothermia may therefore also contribute to the beneficial effect on cellular metabolism Oxygen delivery to dogs in hemorrhagic shock under normothermal conditions does not seem to alter the survival rate Manger *et al* 1962) The ventilatory rate is increased and the oxygen tension in arterial blood is not markedly affected Therefore the oxygen delivery to the tissues will be limited by the blood flow At reduced body temperature the fluid shift from the interstitial compartment to plasma after hemorrhage is smaller indicating a lower dehydration of the interstitial tissue Therefore if the depression of respiratory rate caused by hypothermia and the concomitant respiratory acidosis Drucker Kingsbury and Graham 1962 is prevented by artificial respiration a slightly better oxygen exchange to the tissues may occur

Conclusions

As judged from the ability of single skeletal muscle cells to actively reaccumulate potassium during in vitro incubation it appears that hypothermic treatment after induced hemorrhagic shock can considerably decrease cellular hypoxic injury Hemorrhagic shock under normothermal conditions, on the other hand results in markedly decreased cellular potassium content and impaired active potassium transport during consequent in vitro incubation

The methods used seem promising for the evaluation of the degree of cellular injury incurred under various in vivo conditions and for the evaluation of cellular response to different therapeutic measures

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Potassium Transport in Single Mammalian Skeletal Muscle Cells during in vitro Incubation

By

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Abstract

HALJAMÄE, H. *Potassium transport in single mammalian skeletal muscle cells during in vitro incubation.* Acta physiol. scand. 1970. *78.* 201—212.

Single skeletal muscle cells were isolated after varying intervals of in vitro incubation at 20—22° C from a muscle biopsy of the adductor muscle of dogs. From each isolated fibre 3—4 pieces 70—90 μ long were cut out and analyzed separately. Dry mass was calculated from x ray absorption measurements. The potassium content was determined from x ray fluorescence microanalysis and from ultra micro flame photometric analysis.

During the first 30—40 min of in vitro incubation profound changes in cellular potassium content took place. Cells isolated during the first few min of incubation usually had a lower potassium content than cells isolated after 5 to 10 min. In the period 10 to 25 min after the initiation of incubation the cells started to loose intracellular potassium. This cellular potassium loss was followed by a period of reaccumulation since cells isolated 30 to 45 min after initiating incubation usually had a potassium content as high as that of cells isolated after 5 to 10 min of incubation. When the sodium pump was inhibited by addition of 10^{-3} M ouabain to the incubation medium or if glycolysis was inhibited by 10^{-3} M monoiodoacetate no reaccumulation of intracellular potassium was seen. 10^{-4} M 2,4 dinitrophenol produced a profound rapid loss of cellular potassium. It therefore appears that the reaccumulation of cellular potassium after the period of cellular potassium loss was dependent on the integrity of the sodium pump and a source of energy. The possible basis for the profund cellular electrolyte changes which occur during in vitro incubation are discussed.

Electrolyte transport in mammalian tissues under in vitro conditions has been studied by a variety of different methods. Measurements of uptake and loss of radioactively labelled substances and flame photometric analysis of total tissue electrolytes are the most commonly used techniques. Since pieces of whole tissue are usually used, the results are not completely representative of the main type of cells studied. Rather the results also reflect the content of other types of cells in the tissue as connective tissue cells, nerves, blood vessels and remaining blood cells.

The innervation and the blood supply to a tissue is cut off under in vitro condition. The diffusion of oxygen and nutritive substances to the cells and the removal of metabolites may be affected due to the increased diffusion distances since the ground substance of the tissues is limiting free diffusion (Wiederhielm 1966). A restricted

mobility of the tissue fluid phase may result in local accumulation of substances and change the ion binding capacity of the mucopolysaccharide molecules of the ground substance (Catchpole, Joseph and Engel 1966)

For studies of the mammalian muscle preparations most commonly used rat diaphragm or extensor digitorum muscle the above mentioned problems will have to be considered According to Day (1952), the muscle fascia is limiting free diffusion considerably and also the metabolism of *e g* the mesothelial layers will affect the results obtained with diaphragm preparations (Peterson Beatty and Bocek 1961) Therefore a study of the electrolyte transport of single mammalian muscle fibers could give more information as many of the interfering factors could be avoided In the case of poikolothermic animals single skeletal muscle cells are often used (*e g* Hodgkin and Horowicz 1959) as they are easier to isolate and less vulnerable than single mammalian muscle cells (Honke 1947) The active electrolyte transport characteristics for muscle fibers from poikolothermic animals are however probably not representative of mammalian muscle cells since the latter are much more dependent on aerobic metabolism The aim of the present study was to obtain a continuous record of the electrolyte metabolism of single mammalian skeletal muscle fibers during in vitro incubation in an physiological saline solution

Methods

Dogs of both sexes and mixed breed which weighed 10 to 20 kg were used A muscle biopsy was taken from the adductor muscle of one of the hind legs during Mebumal (pentobarbital sodium anesthesia 30—40 mg/kg During the biopsy sampling precautions were taken to avoid damage to the biopsy caused by overstretching pressure or crush injury The biopsy was immediately rinsed for 10—20 sec in an oxygenated dissection solution and placed in a Petri dish containing the solution The dissection (incubation) solution had the following composition in mM K 5 6 Na 160 Cl 164 Ca 2 2 Mg 0 3 HCO_3 5 9 and glucose 2 8 Before use the solution was bubbled for 10 minutes with a mixture of 95 % O_2 and 5 % CO_2 and pH was adjusted with $NaHCO_3$ to 7 3 In a few experiments metabolic inhibitors were added to solution Ouabain interfering with the efficiency of the sodium pump was used at a concentration of 10^{-3} M monoiodoacetate interfering with glycolysis at 10^{-3} M and 2 4-dinitrophenol interfering with high energy phosphate compound synthesis at 10^{-3} M

Dissection of single skeletal muscle cells

During incubation in the dissection solution at 20—22 C single skeletal muscle fibers were dissected out under a stereo microscope at a magnification of 50 [illegible] × Stainless steel microneedles or very thin pointed tungsten needles obtained by electrical etching in an electrolyte bath (0 6 N NaOH at 30 40 V [illegible] A were used for this dissection Two slightly different dissection procedures were used

A Single cells were isolated in the dissection solution and transferred on the tip of the micro-needle to a clean mylar foil Subsequently the excess dissection solution was immediately aspirated away with a thin pointed micropipette allowing the cells to dry almost immediately (Haljamäe 1967

B Bundles of skeletal muscle fibers consisting of 10 20 fibers were isolated in the dissection solution The bundles were transferred to a clean mylar foil and the adhering dissection solution aspirated away On the mylar foil single fibers were separated from the bundle Several single fibers could be isolated without direct injury or extensive stretching by [illegible] them gently and moving them 0 1 1 mm out from the bundle With this method several cells could be obtained which had the same time period of in vitro incubation The time between taking the biopsy and the isolation of each separate cell was carefully recorded After the isolation of each cell the dissection solution was also exchanged for fresh solution as electrolyte changes or pH changes due to leakage from the cut ends of the biopsy might otherwise [illegible] cellular metabolism The dissection was continued for 40 to 60 min and usually 15 to [illegible] single fibers or groups of fibers were isolated during this time period

was analyzed separately, hence, multiple electrolyte analyses of each muscle fibre were obtained which facilitated the detection of contaminants in occasional samples

Analytical methods

The dry mass of each cut out piece of muscle cell was determined by use of an x ray absorption technique (Rosengren 1959) Soft x-rays in the 8—10 Å range were used The piece of muscle cell was placed on a mylar foil above a 100 μ aperture from the x-ray tube The transmitted radiation was measured by a proportional counter and corrected for background absorption due to the supporting mylar foil The residual absorption attributable to biological tissue was then equoated to a given dry mass by means of a standard curve generated from absorption readings obtained from thin nitrocellulose films, which have approximately the same x ray absorption as biological material The mass per surface unit of these films was determined according to Djurle and Hallen (1953) Duplicate or triplicate mass determinations of each piece of muscle cell were performed The dry mass of the pieces of muscle cells ranged from $5\times10^{-9}-5\times10^{-8}$ g

Two different micro-analytic methods were used for the determination of the potassium content of the cut out pieces of skeletal muscle fibre The methods used were (A) x ray fluorescence microanalysis (Long and Röckert 1963), and (B) ultra micro flame photometry (Haljamäe and Larsson 1968)

A X ray fluorescence microanalysis

The application of this method to the analysis of the potassium content of single skeletal muscle cells has been described in a previous report (Haljamäe 1967) The piece of muscle cell was placed on a mylar foil lying over a 200 μ aperture of a Cosslett-Nixon x ray microscope X rays were generated at 20 kV and 50 μA A constant sector of the fluorescent radiation was taken up by a proportional counter and analyzed by a 256-channel, pulse height analyzer, which could discriminate between the different energy levels of the elements in the sample The radiation intensity at the different energy levels was displayed as a curve on an oscilloscope, and also printed out automatically The peaks of the curve were proportional to the amount of the elements present, which had a fluorescent energy within the part of the energy spectrum analyzed The amount of potassium in the piece of muscle fibre analyzed was calculated from a standard curve

B Ultra micro flame photometry

After their dry mass determination the cut-out pieces of skeletal muscle cell were placed on a quartz glass in a Petri dish The quartz glass was carefully cleaned by soaking in concentrated nitric acid over night and rinsed 10 times with deionized water and ten times with redistilled water before drying under dustfree conditions The pieces of muscle cell on the quartz glass were covered with liquid paraffin (Merck No 7162 "pro injectione Darmstadt Germany) Each piece was extracted for 2 hrs with a known volume usually 70 8 nl of quartz redistilled water or 2 N nitric acid pipetted onto the cell with a calibrated nanoliter quartz pipette The liquid paraffin cover prevented dust contamination and evaporation of the small extraction volumes used. Duplicate or triplicate samples (usually 21 8 nl of the extract from each piece were separately analyzed with the ultra micro flame photometer

A known volume of the extract was pipetted onto the tip of a 100 μ thick platinum-iridium wire attached to a metal wire holder The wire holder was inserted into a port in the flame photometer housing and connected to an electro-magnet On actuation of the magnet the sample was inserted into the flame while simultaneously the integrators of the detector system were activated. The light emitted from the vaporized sample was focussed onto two separate photocells by two collector lens systems with interference filters The filter of the channel for potassium light detection had its transmission maximum at 762 mμ and the channel for sodium light detection had its maximum at 584 mμ The outputs from the two photocells were connected to separate amplifiers and integrators and the integrated values of the potassium and sodium content of the sample were displayed on separate indicators

Results

With both dissection procedures and both analytic methods reproducible changes in cellular potassium content could be demonstrated during in vitro incubation at

mobility of the tissue fluid phase may result in local accumulation of substances and change the ion-binding capacity of the mucopolysaccharide molecules of the ground substance (Catchpole, Joseph and Engel 1966).

For studies of the mammalian muscle preparations most commonly used rat diaphragm or extensor digitorum muscle, the above mentioned problems will have to be considered. According to Day (1952), the muscle fascia is limiting free diffusion considerably and also the metabolism of *e.g.* the mesothelial layers will affect the results obtained with diaphragm preparations (Peterson, Beatty and Bocek 1961). Therefore a study of the electrolyte transport of single mammalian muscle fibers could give more information as many of the interfering factors could be avoided. In the case of poikilothermic animals single skeletal muscle cells are often used (*e.g.* Hodgkin and Horowicz 1959) as they are easier to isolate and less vulnerable than single mammalian muscle cells (Honke 1947). The active electrolyte transport characteristics for muscle fibers from poikilothermic animals are, however, probably not representative of mammalian muscle cells since the latter are much more dependent on aerobic metabolism. The aim of the present study was to obtain a continuous record of the electrolyte metabolism of single mammalian skeletal muscle fibers during in vitro incubation in an physiological saline solution.

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Dissection of single skeletal muscle cells

During incubation in the dissection solution at 20–22 C single skeletal muscle fibers were dissected out under a stereo microscope at a magnification of 50–80 ×. Stainless steel microneedles or very thin pointed tungsten needles (obtained by electrical etching in an electrolyte bath (0.5 N NaOH) at 30–40 V, 1–2 A) were used for this dissection. Two slightly different dissection procedures were used.

A. Single cells were isolated in the dissection solution and transferred on the tip of the micro-needle to a clean mylar foil. Subsequently the excess dissection solution was immediately aspirated away with a thin pointed micropipette allowing the cells to dry almost immediately (Haljamäe 1967).

B. Bundles of skeletal muscle fibers, consisting of 10–20 fibers, were isolated in the dissection solution. The bundles were transferred to a clean mylar foil and the adherent dissection solution aspirated away. On the mylar foil single fibers were separated from the bundle. Several single fibers could be isolated without direct injury or extensive stretching by lifting them gently and moving them 0.1–1 mm out from the bundle. With this method several cells could be obtained which had the same time period of in vitro incubation. The time between taking the biopsy and the isolation of each separate cell was carefully recorded. After the isolation of each cell the dissection solution was also exchanged for fresh solution as electrolyte changes or pH changes due to leakage from the cut ends of the biopsy might otherwise affect cellular metabolism. The dissection was continued for 40 to 50 min and usually 15 to 20 single fibers or groups of fibers were isolated during this time period.

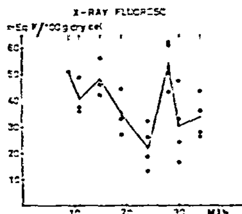


Fig 2 Cellular potassium content changes with time during in vitro incubation as obtained from x ray fluorescence analysis

the difference in the potassium content of such simultaneously isolated cells is small (in this case average 2.4 meq/100 g dry muscle cell) and independent of when the cells were isolated. Altogether 33 such comparisons of the potassium content of cells isolated after the same time period of in vitro incubation were performed. The average difference in the potassium content was 4.9 meq 100 g between such cells this is considerably lower than the cellular potassium shifts observed with time. 20—25 meq/100 g

Fig 4 and 5 illustrate the potassium shift curves representing the average values for all the muscle cells isolated within successive 5-min intervals. Fig 4 represents results from x ray fluorescence analyses of 32 cells 105 different cut out pieces from 4 dogs. The average potassium value of cells isolated 0—10 min after biopsy 51 0 meq/100 g dry muscle cell is higher ($P<0.02$) than the average value 30 6 meq 100 g for cells isolated 16—20 min after biopsy. Cells isolated after 20 min of incubation or more have a higher potassium content than the lowest value at 16—20 min although no clearly significant difference is demonstrable 16—20 min value vs 21—25 min value $P<0.2$ 16—20 min value vs 26—30 min value $P<0.1$ Fig 5 de-

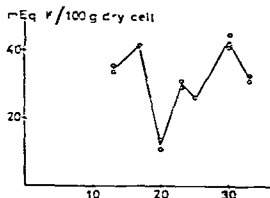


Fig 3 Potassium content of cells isolated from one muscle biopsy after the same time periods of in vitro incubation

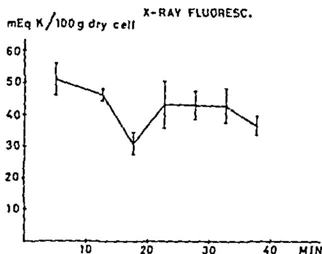


Fig 4 Averaged potassium content changes of cells from 4 dogs The x ray fluorescence microanalytic values of cells isolated within 5 min intervals during the in vitro incubation grouped S E M values represented by vertical bars

plays the corresponding average values for 116 cells (272 analyzed cut out pieces) from 12 dogs analyzed by ultra micro flame photometry A curve similar to Fig 4 is obtained but in this case the lowest value was not seen until after 31—35 min of incubation The average potassium value of cells isolated after 6—10 min 43 4 meq 100 g is higher ($P<0\ 02$) than the lowest value 30 1 meq/100 g for cells isolated after 31—35 min This latter value is on the other hand lower ($P<0\ 01$) than the average value, 43 8 meq/100 g of cells isolated after 41—45 min

There was however a marked variation among cells from different dogs in the time periods for potassium loss and reaccumulation during the in vitro incubation (*cf* Fig 1) These variations tend to make the potassium shifts with time less significant if the values are averaged as in Fig 5 If instead the first value of completed cellular potassium reaccumulation is taken as a fixed time and the potassium content of cells isolated within successive 5 min intervals before and after this time are grouped (Fig 6) then such temporal variations are partially excluded The curve for the average potassium changes is then more in accord with those from individual

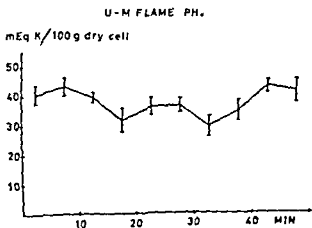


Fig 5 Averaged ultra micro flame photometrically determined potassium content changes with time of in vitro incubation of cells from 12 dogs Cells isolated within 5 min intervals of incubation grouped and S E M values given as vertical bars

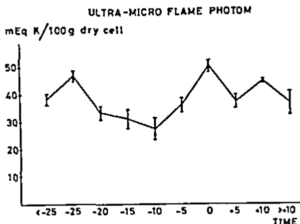


Fig 6 The values for completed potassium reaccumulation taken as fixed time (zero-time value) The potassium content values for cells isolated within 5 min intervals prior and after this time are grouped and the S E M values given as vertical bars In this way variations between the temporal pattern of potassium changes in cells from different experimental animals can be partly excluded

experiments (*cf* Fig 1, 2, 3, and 7) Cells isolated more than 25 min before completed potassium reaccumulation have an average potassium content of 38 7 meq/100 g which is lower than that in cells isolated within the following 5 min interval (47 0 meq/100 g, this difference not significant $P<0\ 1$) The latter value is significantly higher ($P<0\ 01$) than all the average values for cells isolated between 5—20 min before completed potassium reaccumulation The zero time value of completed potassium reaccumulation, 50 4 meq/100 g, is also significantly higher ($P<0\ 001$) than all the average values of cells isolated 5—20 min prior to that Also if instead the lowest value for potassium during the period of cellular potassium loss is taken as a fixed time and the potassium content of cells isolated within 5-min intervals from this time are similarly grouped, highly significant differences will be obtained between the zero time value and those of cells isolated before and after this time

Evidence indicating that the potassium shifts observed in skeletal muscle cells under in vitro conditions were active and energy dependent was obtained from experiments in which metabolic inhibitors were added to the incubation medium (Fig 7) Data for this Fig came from 4 biopsies taken from the same dog The control biopsy was taken last to detect if the prolonged period of anesthesia required would affect the electrolyte shift curve As can be seen from Fig 7 the potassium changes in cells from the control biopsy were in agreement with the previous results When 10^{3} M ouabain was added to the incubation medium the initial potassium values were somewhat lower but a slight reaccumulation was seen After 20 min of incubation the intracellular potassium content decreased rapidly in the presence of ouabain 10^{3} M monoiodoacetate had a similar effect Both the marked loss of intracellular potassium normally seen after 10—20 min of incubation and its reaccumulation were absent Instead monoiodoacetate produced a continuous loss of intracellular potassium 10^{3} M 2 4-dinitrophenol had the most marked effect on the potassium content of the skeletal muscle cells causing a very rapid loss The cells also became more vulnerable at the dissection and after 25 min of incubation it was no longer possible to isolate cells of normal appearance

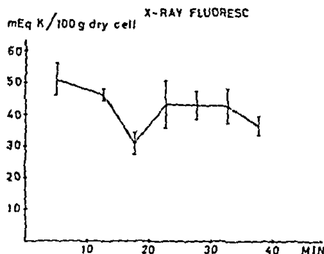


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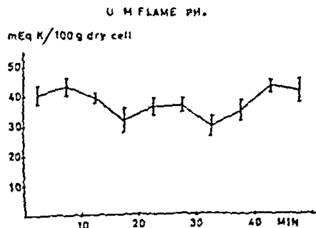


Fig 5 Averaged ultramicro flame photometrically determined potassium content changes with time of in vitro incubation of cells from 12 dogs Cells isolated within 5 min intervals of incubation grouped and S E M are given as vertical bars

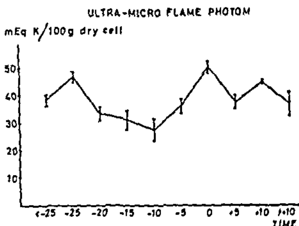


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experiments (*cf* Fig 1 2, 3 and 7) Cells isolated more than 25 min before completed potassium reaccumulation have an average potassium content of 38 7 meq/100 g which is lower than that in cells isolated within the following 5 min interval (47 0 meq/100 g, this difference not significant $P<0.1$) The latter value is significantly higher ($P<0.01$) than all the average values for cells isolated between 5 20 min before completed potassium reaccumulation The zero-time value of completed potassium reaccumulation 50 4 meq/100 g is also significantly higher ($P<0.001$) than all the average values of cells isolated 5–20 min prior to that Also if instead the lowest value for potassium during the period of cellular potassium loss is taken as a fixed time and the potassium content of cells isolated within 5 min intervals from this time are similarly grouped highly significant differences will be obtained between the zero-time value and those of cells isolated before and after this time

Evidence indicating that the potassium [illegible] observed in skeletal muscle cells under in vitro conditions were active and energy dependent was obtained from experiments in which metabolic inhibitors were added to the incubation medium (Fig 7) Data for this Fig came from 4 biopsies taken from the same dog The control biopsy was taken last to detect if the prolonged period of [illegible] would affect the electrolyte shift curve As can be seen from Fig 7 the [illegible] in cells from the control biopsy were in agreement with the [illegible] When 10^{-3} M ouabain was added to the incubation medium the [illegible] were somewhat lower but a slight reaccumulation was seen After [illegible] tion the intracellular potassium content decreased rapidly in the presence of [illegible] 10^{-3} M monoiodoacetate had a similar effect Both the marked [illegible] potassium normally seen after 10—20 min of incubation and [illegible] absent Instead monoiodoacetate produced a continuous loss of potassium [illegible] tassium 10^{-3} M 2 4 dinitrophenol had the most marked effect on the potassium content of the skeletal muscle cells causing a very rapid loss The cells [illegible] vulnerable at the dissection and after 25 min of incubation it was no longer [illegible] to isolate cells of normal appearance

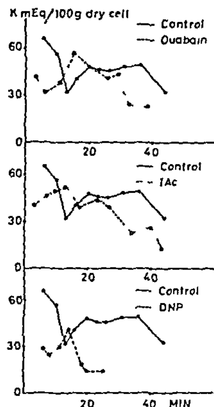


Fig 7 Effects of metabolic inhibitors on the potassium content of single skeletal muscle fibers during in vitro incubation

Discussion of the techniques employed

With the micro-techniques employed cells could be isolated after relatively short periods of incubation Therefore an almost continuous check of the electrolyte content of cells from the same biopsy during in vitro incubation was possible The muscle fibers were always isolated from the surface of the biopsy Therefore the intracellular ion concentrations measured would not be markedly affected by diffusion restrictions and possible interstitial accumulation of ions and metabolites Several cells could be isolated after the same time period of incubation which made it possible to evaluate the extent to which cellular injury produced during the isolation procedures affected the electrolyte values Variability in the results due to contamination of the samples during the different steps of the analysis was reduced by analyzing several cut out pieces from each cell separately If one piece had been heavily contaminated this was readily apparent The incubation medium was exchanged for fresh solution every few min therefore changes in the external fluid composition due to leakage from the damaged cells at the cut ends of the biopsy were prevented

Since the cells were not rinsed after isolation the cellular values obtained were slightly affected by adhering incubation medium Relatively reproducible contamination of the samples due to incubation fluid adherance was however preferred to variations resultant from cellular damage and possible dust contamination which

might be incurred during an additional step in the sample handling when using a rinsing procedure The effect of such adhering fluid on the potassium values was small but the sodium values from ultra micro flame photometry were not representative of the intracellular sodium content The measured sodium content was usually 30—50 meq/100 g dry muscle cell True intracellular sodium is probably considerably lower, therefore most of the measured sodium represents adhering medium The effect of such adherance on the cellular potassium values will be an overestimation of 1—2 meq/100 g (as Na in the medium 160 mM K 5 6 mM) This overestimation of the intracellular potassium content due to the potassium in the adhering incubation medium was reduced by the cellular content of calcium magnesium and phosphate which has been shown to decrease the potassium readings from ultra micro flame photometry by 2—4 % (Keesey 1968, Haljamäe and Larsson 1968) In fact the potassium values obtained from ultra micro flame photometry seem to be representative of the expected intracellular potassium content Within the sensitivity range needed for ultra micro flame photometric analysis of the nl volumes of the extracts (10^{-10}—10^{-11} M) there was high reproducibility during analysis of known nl volumes of standard solutions The average variations were $\pm$1 2 % for potassium and $\pm$ 1 3 % for sodium (Haljamae and Larsson 1968)

With the x ray fluorescence micro method it was possible to analyze down to 10^{-11} g of potassium with accuracy The application of this method to potassium analysis of biological material has certain disadvantages due to interference from other elements Chlorine has a fluorescent energy close to that of potassium and complete separation of the energy peaks for these two elements was not possible with the equipment used Since the chloride content of the extracellular fluid and the incubation fluid was high and the cells also contain chloride slight chloride contamination and resultant increase of the measured potassium readings always occurred Therefore corrections for chloride interference had to be made during the calculation of the cellular potassium content A correction factor for this interference had to be calculated and was as high as 0 53 (Haljamae 1967) Calcium also has a fluorescent peak close to that of potassium and interferred slightly with the potassium measurements (Haljamae and Rockert 1969) Therefore in spite of the usage of the correction factor of 0 53 the calculated cellular potassium values from x ray fluorescence micro-analysis were slightly higher than those obtained from ultra micro flame photometry

General discussion

During isolation and in vitro incubation of mammalian muscle preparations an initial loss of cellular potassium has been demonstrated previously (*e g* Calkins Taylor and Hastings 1954) An initial loss followed by reaccumulation has also been shown to occur in other types of mammalian tissues *e g* guinea pig taenia coli preparations (Goodford and Hermansen 1961) in rat aorta preparations (Dawkins and Bohr 1960) and also in non mammalian tissue (Giacobini Hovmark and Kometiani 1967, crayfish sensory neurons) The initial loss of intracellular potassium

and gain of sodium has usually been considered to be resultant from cellular injury caused during the manipulations involved in the isolation of the tissue. Active metabolism is thought to compensate for this loss during prolonged incubation.

The present results are not completely in agreement with the concept that tissue damage during isolation is the main reason for these changes of cellular potassium. The marked loss of cellular potassium does not strat until after 15 min of in vitro incubation of the skeletal muscle fibres. The slightly lower potassium content of cells isolated shortly after the incubation is probably due to tissue irritation at the taking of the biopsy but usually this seems to be rapidly compensated for as evidenced by a slight increase of the potassium content during the following 10 min of incubation. The long time lag before the potassium loss starts is more suggestive of a change in cellular metabolism under the in vitro conditions. Therefore the observed cellular potassium changes during in vitro incubation will be considered on the basis of possible effects of incubation on cellular metabolism.

The content of high-energy phosphate compounds decreases in a tissue during its isolation. For instance, Peterson, Beatty and Bocek (1961) found markedly less creatine phosphate in rat adductor muscle fibre bundles which were frozen during dissection than in those frozen in situ. Beviz and Svedmyr (unpublished report) have recently shown that there is a tenfold decrease in the adenosinetriphosphate content of gastrocnemius muscles of rats isolated at room temperature as compared to those frozen in situ. Such a decrease in the concentration of high-energy phosphate compounds might mean that the energy available to the cells at the beginning of incubation is limited. From the potassium content values of cells isolated during the first 10—15 min of incubation, it seems reasonable to assume that the high-energy phosphate compounds remaining at the beginning of incubation are sufficient to keep up a high cellular potassium content or even to give rise to a slight net uptake of potassium. The speed of phosphorylation in the freshly isolated tissue might, however, be too slow to cope with the demands of the sodium pump during prolonged in vitro incubation; therefore the cells start to leak potassium. The temperature of 20—22 °C of the incubation medium will probably also make phosphorylation less efficient. After some time the phosphorylation efficiency seems to have increased and enables the cells to reaccumulate intracellular potassium. This reaccumulation of cellular potassium is prevented by metabolic inhibitors. Ouabain, which interferes with the active movements of potassium and sodium (Glynn 1964), produces a rapid loss of cellular potassium after about 30 min of incubation as compared to the control biopsy. Kostyo and Schmidt (1963) also used 10^{-4} M ouabain during in vitro incubation of rat diaphragm and found a profound reduction of cellular potassium after prolonged incubation. The initial increase of the cellular potassium content in the presence of ouabain might be due to a slowly developing inhibition of the sodium pump which is not complete until after 20—30 min. The results from the effect of iodoacetate on the cellular potassium content are in agreement with the above discussion of available cellular energy at the beginning of the incubation, i.e. that the initial high-energy phosphate content is sufficient to keep up a high cellular potassium

content during the first 10 to 15 min of incubation The inhibitory effect of iodoacetate on glycolysis develops slowly and a muscle stimulated in the presence of iodoacetate only slowly loses its excitability (see Webb 1966) During the first 15 min of incubation in the iodoacetate containing medium a slow net uptake of potassium is seen in the skeletal muscle fibers indicating that remaining high energy phosphate can provide enough energy for the sodium pump After these first 15 min of incubation there is a slow continuous loss of potassium in agreement with cellular energy depletion The rapid effect of 2 4 dinitrophenol on the potassium content of the skeletal muscle fibres is probably due to the inhibiting effect of the drug on adenosinetriphosphate synthesis together with increased rates of hydrolysis of both creatine phosphate and adenosinetriphosphate (Slater 1963) Abood Koketsu and Noda (1961) found that if frog muscle was incubated in 10^{-3} M 2 4 dinitrophenol containing solution the tissue content of creatine phosphate was reduced by 97 % and that of adenosinetriphosphate by 77 % These authors also reported that dinitro phenol at a concentration of 0 1 mM produced a rapid decrease of the resting potential of frog muscle

Other factors could also contribute to the changes of cellular potassium content during in vitro conditions such as the deprevation of the muscular tissue of its innervation and hence its normal neurohumoral supply and level of basal tonus Dockery Kernan and Tangney (1966) have shown that intact innervation considerably increased active potassium and sodium transport of rat extensor digitorum and soleus muscles during recovery from sodium enrichment Isoprenalin was also observed to increase active transport The deprevation of cells from these factors during the incubation may contribute to the time lag until potassium reaccumulation begins and the efficiency of the pump or phosphorylation adjust to the changed conditions

It may be concluded that during the first 40 to 50 min of in vitro incubation under the experimental conditions used there are profound changes in net cellular potassium fluxes of single mammalian skeletal muscle cells It seems likely that these changes can be related to the availability of high energy phosphate compounds The connection of these in vitro electrolyte shifts with available energy has also been demonstrated in a separate report (Haljamae 1969) in which normal cellular metabolism was interferred with in vivo (hemorrhagic shock before the in vitro incubation The main advantage of the present experimental approach to the effect of in vitro incubation on cells is that cells from the same biopsy can be isolated within short time intervals Therefore variations in between different experimental animals can be excluded and the technique makes it possible to get an almost continuous record of the cellular events It would however also be valuable to compare the temporal electrolyte changes during in vitro incubation with electron microscopic studies of possible changes in cellular ultrastructure Such a study would reveal if there are concomitant changes in cellular morphology which might contribute to or further explain the observed electrolyte changes

An investigation of the content of high energy phosphate compounds and the

metabolism of muscles under the same conditions as used in the present experiment is carried on and will be presented in a later report

This work was supported by grants from the Medical Faculty, University of Goteborg Sweden and the Swedish Research Council (Project B 69 17X 127 05) I am indebted to Dr David C Wood for valuable help with the preparation of the manuscript, to Dr A Hamberger for valuable discussions and to Mrs Anita Modh Miss Ingela Lestring and Mrs Britt Marie Otterstedt for skilful technical assistance

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The Evolution of Vascular Smooth Muscle Responses to Histamine and 5-Hydroxytryptamine

III. Manifestation of dual actions of either amine in reptiles[2]

By

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Received 29 March 1969

Abstract

REITE, O B *The evolution of vascular smooth muscle responses to histamine and 5 hydroxytryptamine III Manifestation of dual actions of either amine in reptiles* Acta physiol scand 1970 *78* 213—231

Studies on the vascular smooth muscle responses to histamine and 5 hydroxytryptamine and on the distribution of these amines in different tissues were performed in several species of reptiles The responses to adrenaline noradrenaline and acetylcholine were studied for comparison Vascular actions were recorded both *in vivo* as changes in systemic arterial blood pressure and *in vitro* as changes in vascular resistance in perfused preparations The specificity of the action of each drug was evaluated by means of pharmacological blocking agents Tissue levels of histamine and 5-hydroxytryptamine were determined spectrofluorometrically Fluorescence microscopy was used for histochemical localization of the histamine stores The results indicate that histamine and 5 hydroxytryptamine exert dual actions (inhibitory and stimulatory) on reptilian vascular smooth muscles which have thus acquired response patterns towards these amines similar to those found in mammals Inhibitory as well as stimulatory actions of adrenaline noradrenaline and acetylcholine are also present in reptiles Levels of 5 hydroxytryptamine are apparently low in most tissues whereas many species show very high tissue levels of histamine Histamine except that of the stomach, is mainly located in tissue mast cells and blood basophils Nutritional dependent variations in mast cell number are parallelled by variations in tissue histamine content By comparing the present results with those previously obtained in jawless vertebrates fish and amphibians a general outline of the evolution of vascular actions and tissue stores of histamine and 5 hydroxytryptamine is presented

Two preceding reports dealt with the actions of histamine and 5 hydroxytryptamine on vascular smooth muscles in jawless vertebrates fish and amphibians (Reite 1969 a b) Evidence was presented that only stimulatory actions are present in fish whereas a further evolutionary step is reached by the appearance of inhibitory actions of

[1] Some of the experiments in the American species were performed during a stay at the Department of Physiology and Biophysics University of Kentucky Lexington, Kentucky U S A

[2] Preliminary reports on parts of this work were made at the Fall Meeting of the American Physiological Society Los Angeles August 1965 and at the Annual General Meeting of Stockholm Physiological Society Stockholm November 1967

TABLE 1 Exper mental an mals

Spec es	Number of an mals	App ox ma body weigh * (kg
Greek torto se (*Te tudo hermann*)	18	0 ?—0 8
Iberian land torto se (*Te udo gra a*	6	0 4—0 (
European pond terrap n (*Emy o b cula s*	18	0 3—0
Span h terrap n (*Clemmys ca p a l b o a*	14	0 1—0 ?
Amer can pond terrap n (*P eudemys cr pla*	10	0 2—1.2
European green l zard (*La erta r d*	12	0 015—0 0
N le varan (*I a anu n lo icu*	3	? 5—4 5
European common per *I pe a beru*	20	0 0 0 08
European wa er snake (*Nat x na x*	15	0 05—0 15
Amer can wa er snake *Na x fedon fas ata*	4	0 2 0 3
Spectacled ca man (*Ca man crocod lus*	13	0 08—0 2

* *Es ma ed af er we gh ng of a few an mals from each spec es*

5 hydroxytryptamine in amphibians The present work discussing results obtained during experiments in reptiles is the final report in this series of studies on the pharmacological evolution of vertebrate vascular smooth muscle It will reveal that the dual response pattern which prevails in mammals ilator as well as n latory actions f both h stamine and 5 hydroxytryptamine also characterises the

Procedure for blood pressure recording Effects of drugs on arterial blood pressure were studied in all species except the European green lizard The animals were equipped with one arterial and one venous catheter Cannulation was performed under urethane anesthesia, induced and maintained by intraperitoneal administration of about 0 8 ml of a 25 % solution per 100 g b w, followed by supplementary doses of 0 2 ml of the same solution when required In turtles, lizards and caimans, the arterial catheter was introduced into the carotid artery, whereas in snakes it was introduced into the posterior part of the dorsal aorta (always in central direction) Correspondingly, the venous catheters were introduced into the jugular vein or an abdominal vein All catheters had been connected to 3 way stop-cocks and filled with heparinized physiological solution prior to the cannulations

After connecting the arterial catheter to the pressure recording system (Statham pressure transducer (P 23 AA) and Sanborn dynograph), blood pressure was continuously recorded throughout the observation period, with the animal still anesthetized Blood pressure effects of drugs were usually studied after i v administration, but the i a route (through the arterial catheter) was also used for comparison In some of the American pond terrapins incisions in the neck which had been made for access to the blood vessels were sutured, and the animals were allowed to recover from anesthesia before the experiment with drug injection was started In the Nile varan, experiments were performed both during and after recovery from anesthesia

The surgical part of the procedure for blood pressure recording was usually accomplished in less than 1/2 hr The observation period during injections of various drugs lasted for about 1/2—3 hrs in anesthetized animals, and from 1—5 hrs in those which had recovered from anesthesia Unanesthetized animals were always studied within the first 24 hrs of implantation of catheters The animals were sacrificed immediately after completion of the experiment *In vivo* experiments were performed at ambient temperatures of 10—12° C or 18—22° C

Perfusion experiments Artificial perfusion of the blood vessels in the hind part of specimens from all species except the Iberian land tortoise and the American water snake was performed with physiological solution Animals to be used in the perfusion studies were killed by decapitation The dorsal aorta was cannulated in peripheral direction, and abdominal veins were cut open to secure free outflow The perfusion technique was principally similar to that previously described for fish (Reite 1969 a) Effects on vascular smooth muscle from the different pharmacological agents were recorded as changes in perfusion pressure at constant rate of flow The flow rate was maintained by using a peristaltic pump, and the perfusion pressure was initially adjusted to a level of 20—40 mm Hg The pressure recording system was the same as that used for recording arterial blood pressure in *in vivo* experiments Drugs were added to the perfusion fluid reservoir or injected as single doses into the tubing of the perfusion circuit between the pump and the vascular bed Ambient temperatures and temperatures of the perfusion fluid were within the range of 18—22° C The observation period in perfusion experiments was 1—6 hrs

chloride compound 48/80 (condensation product of p methoxyphenethylmethylamine with formaldehyde cf Paton 1951), polymyxin B sulfate, pyrilamine maleate chlorpheniramine maleate

bacterial assay units, and those of the other drugs as weight of the compounds in the form listed above All drugs were dissolved in physiological solution and the volumes applied both for intravascular injection and for introduction into the perfusion circuit during artificial perfusion were 0 02—1 ml The effects caused by the injections *per se* were checked by control

histamine, and the procedure outlined by Udenfriend (1964) for 5 hydroxytryptamine

Histological observations These observations were made by means of a Leitz Ortholux microscope equipped for fluorescence microscopy and photomicrography Mast cells and

basophil leucocytes were studied in blood and tissues from all species. Studies were ma'e on fresh tissue spreads (small pieces of tissue stretched on an object glass) stained wi h an aqueous solution of toluidine blue (0 1 %), on tissue spreads fixed in 96 % ethyl alcohol and subsequently stained with alcoholic thionin (0 1 g thionin per 100 ml 80 % ethyl alcohol and on tissue spreads directly stained with the alcoholic solution of thionin Blood smears were air dried at 30—35° C before fixation and staining Histochemical localization of histamine was achieved by applying the preparation procedure and filter arrangement described by J h lin and Shelley 1966) Treatment of the tissues with buffered physiological solution (pH ll was often unnecessary for development of satisfactory fluorescence upon addition of o-phthala delyde The o-phthalaldehyde was dissolved either in xylene or in toluene but best res n were obtained with xylene

Results

Initial levels of heart rate and blood pressure

At 18—22 C, the frequency of heart beats in reptiles as seen before administration of any vasoactive agents ranged from about 20—80 per minute but frequencies within the lower or middle part of this range were most common The corresponding values for arterial blood pressure were 25—60 mm Hg at systole and 20—50 mm Hg at diastole Blood pressure levels in turtles and caimans were generally lower than in lizards and snakes At temperatures of 10—12° C the heart beats were approximately half as frequent as at 18—22° C whereas the differences in blood pressure were less pronounced Both heart rate and blood pressure showed spontaneous variations even when the animals were in deep anesthesia

Effects of pharmacological agents

Turtles In tortoises the blood pressure effects produced by histamine (0 5—10 µg varied from purely depressor—with only a slight increase in pulse pressure as was common in the Iberian land tortoise (Fig 1)—to a transient depressor effect fo owed by a weak pressure rise and a somewhat stronger increase in pulse pres re The latter response pattern was the more common in the Greek tortoise In p nd errapins the most conspicuous effect of histamine (0 2 10 µg) was a p wer l

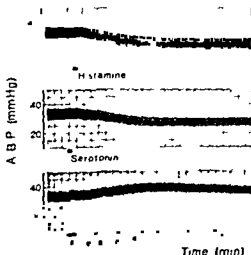


Fig 1 Blood pressure responses of the land tortoise *Testud graeca* to i travenously administered histamine 2 µg upper tracing 5-hydroxytryptamine serotonin 1 µg middle tracing and adrenaline 10 µg lower tracing

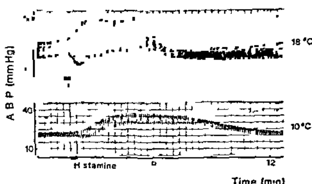


Fig 2 Cardiovascular effects of histamine (10 μg) in two American pond terrapins (*Pseudemys scripta*) studied at different temperatures (10° C and 18° C) Both animals were anesthetized and were among the largest specimens of this species Note that at the higher temperature the effects on the heart were dominating (upper tracing), while the presence of stimulatory actions on vascular smooth muscle became more evident and caused a marked rise in blood pressure at the lower temperature

stimulatory action on the heart, causing a strong increase in the force of the heart beat without any significant change in heart rate (Fig 2) This action on the heart made it difficult to evaluate the effects on blood vessels However, at the higher temperatures (18—22° C), there was a decrease in blood pressure after the action on the heart seemed to have ceased (Fig 2, upper tracing) while at 10—12° C the blood pressure rise was of such magnitude that it would be difficult to explain without the presence of vasoconstriction (Fig 2, lower tracing) Intra arterially administered histamine apparently produced less marked effects on the heart than histamine given intravenously whereas the concomitant rise in blood pressure was enhanced Unanesthetized specimens of the American pond terrapin showed cardiovascular responses to histamine similar to those studied under anesthesia In the Spanish terrapin, the effects of histamine were principally the same as in the pond terrapins although the circulatory system of this species seemed to be less sensitive

Apart from its effects on the heart and blood vessels, histamine usually induced respiratory movements in all species of turtles Other muscular movements, including swimming with the hind legs in terrapins lying on their backs were also encountered These movements often disturbed recording of blood pressure changes following administration of histamine

The vascular effects of 5 hydroxytryptamine (0 2—5 μg) were similar in all species of turtle and showed a strong decrease in blood pressure (Fig 1) Muscular movements were also seen after injection of 5 hydroxytryptamine, but were less common than after histamine

Low doses of compound 48/80 (20—75 μg) and polymyxin B (500—1000 units) had only slight effects A weak decrease in blood pressure was occasionally present in the tortoises and the Spanish terrapin, while in pond terrapins a transient pressure rise was observed However, when the dose of compound 48/80 was raised to 100—400 μg an increase in both the force of the heart beat and in blood pressure could be elicited in the pond terrapins Such injections were usually followed by muscular movements which disturbed the pressure recording

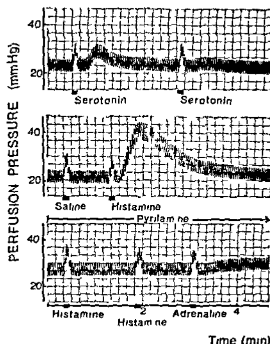


Fig 3

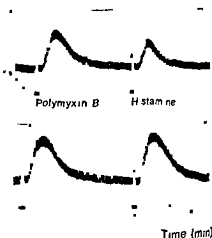


Fig 4

Fig 3 Perfusion of the blood vessels of the hind part of *Pseudemys scripta* There was a marked rise in perfusion pressure following the first of two successive injections of 5 hydroxytryptamine (serotonin 3 μg upper tracing) but tachyphylaxis towards this agent was already present when the second dose was given The middle tracing demonstrates the absence of response to physiological solution (saline) and the strong pressor response to histamine (4 μg) The bottom tracing was obtained after pyrilamine (15 μg/ml) had been added to the perfus on flu d causing blockade of the response to histamine (15 μg and 20 μg) whereas the pressor effect of adrenaline (3 μg) was retained The transient rise in perfusion pressure associated with each njection is due to the injected fluid volume and appears in all tracings from perfus on ex periments

Fig 4 Effects of polymyxin B (1500 units) histamine (1 μg and 3 μg) and compound 48/80 (20 μg) on vascular resistance in the perfused hind part of the European pond terrapin (*Emys orbicularis*) Note the similarities in the responses to these agents The animal was killed 20 hours prior to the experiment and in the meantime the preparation was stored at 4—6° C.

Perfused preparations showed an increase in vascular resistance after administration of histamine and 5 hydroxytryptamine as well as after compound 48/80 and polymyxin B (Fig 3 and 4) With respect to sensitivity to these drugs the species differences were large When histamine was introduced into the perfusion circuit the dose necessary to produce a measurable increase in resistance in preparations from the Greek tortoise was 10—20 μg in the Spanish terrapin 1—2 μg and in the pond terrapins less than 0 5 μg Generally the species which showed low sensitivity towards histamine also showed low sensitivity towards 5 hydroxytryptamine but for the latter agent the dose necessary to elicit a measurable response in all species was about twice the corresponding dose of histamine Tachyphylaxis towards 5 hydroxytryptamine often developed In a preparation from the European pond terrapin

perfusion was performed through the left lateral aorta after ligating the right thus including the vascular beds of many internal organs besides those of the hind part In this preparation both histamine and 5 hydroxytryptamine produced an initial marked decrease in resistance, followed by an increase Compound 48/80 and polymyxin B were tested in doses of 10—100 μg and 1000—2500 units, respectively For these substances the response was also very strong in pond terrapins (Fig 4) and weak in the Greek tortoise while in the Spanish terrapin it was intermediate Muscular movements were often seen in the perfused preparations These movements could be prevented by adding low concentrations of urethane or tubocurarine to the perfusion fluid or by storing the preparation overnight at 4—6° C, which did not change the nature of the response to any drug

The influence of pharmacological blocking agents on the responses to histamine 5 hydroxytryptamine compound 48/80 and polymyxin B was studied in the Spanish terrapin *in vivo* and in perfused preparations from pond terrapins Chlorpheniramine (0 1—0 3 mg) changed the blood pressure response of the Spanish terrapin to histamine by enhancing the depressor component, probably through a more effective blockade of the counteracting stimulatory component Blood pressure responses to 5 hydroxytryptamine were abolished by methysergide (0 5 mg) Methysergide (2—5 μg/ml) also abolished the effects of 5 hydroxytryptamine on vascular resistance in perfused preparations whereas the effects of histamine compound 48/80 and polymyxin B on perfused preparations were diminished or completely blocked by pyrilamine (5—15 μg/ml)

No marked species differences were found in the vascular responses of turtles to adrenaline (2—20 μg) noradrenaline (2—20 μg) isoproterenol (1—5 μg) and acetylcholine (0 2—15 μg) Adrenaline and noradrenaline invariably caused a rise in arterial blood pressure and increased the perfusion pressure during artificial perfusion It was noticed throughout this work however that the pressor responses to adrenaline and noradrenaline in reptiles seemed less marked than those obtained both in bony fish and in amphibians Isoproterenol produced a decrease in blood pressure and an increase in heart rate but had negligible effects on perfused preparations Low doses of acetylcholine *in vivo* had depressor effects only whereas higher doses in addition produced a decrease in heart rate Perfused preparations always responded to acetylcholine by an increase in resistance Pressor effects of adrenaline and noradrenaline could be completely abolished by phenoxybenzamine or phentolamine Propranolol and atropine blocked the effects of isoproterenol and acetylcholine respectively All these blocking agents were applied in doses of about 0 1—0 4 mg per 100 g b w in *in vivo* experiments and in concentrations of 2—5 μg/ml in perfusion experiments

Lizards and snakes The responses of lizards and snakes showed many similarities At 18—22° C histamine (0 5—15 μg) always produced a decrease in blood pressure (Fig 5 and 6) while the effect at 10—12° C which was studied in water snakes only was negligible Effects of 5 hydroxytryptamine (2—15 μg) were slight in the European common viper (Fig 6) On the blood pressure of water snakes and the

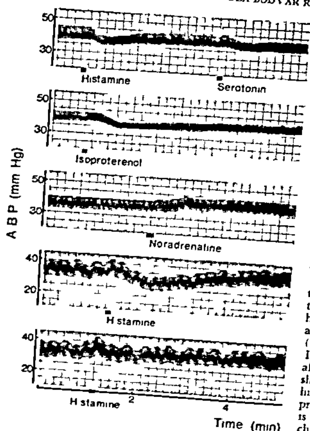


Fig 5 Effects of drugs injected iv on the blood pressure of the Nile varan (*Varanus niloticus*) The three upper tracings, obtained in a lightly anesthetized animal depict the responses to histamine (4 μg), 5-hydroxytryptamine (serotonin, 4 μg), isoproterenol (5 μg) and noradrenaline (10 μg) The two lower tracings were obtained after recovery from anesthesia and show that the depressor response to histamine (15 μg) which is normally present (second tracing from bottom is abolished after injection of 2 mg of chlorpheniramine (bottom tracing)

Nile varan this agent showed a similar but less potent depressor effect than histamine (Fig 5) Neither histamine (5—20 μg) nor 5-hydroxytryptamine (5—20 μg) had any marked effect on perfused preparations although after administration of high doses (20—50 μg) a weak increase in vascular resistance was generally seen Compound 48/80 produced a moderate increase in resistance The influence of pharmacological blocking agents on the responses to histamine and 5 hydroxytryptamine was studied in the Nile varan Blood pressure effects of histamine were completely blocked after injection of diphenhydramine (25 mg) or chlorpheniramine (1—2 mg Fig 5) Blockade of the depressor effects of 5-hydroxytryptamine on the other hand could not be achieved with methysergide (1 mg) Vascular responses to adrenaline noradrenaline isoproterenol and acetylcholine were principally similar to those obtained in turtles and the responses could also be abolished by means of the same blocking agents Tracings from blood pressure recordings during administration of isoproterenol and noradrenaline are shown in Fig 5 and 6

Crocodilians The spectacled caiman was the only species studied from this order of reptiles Histamine (1—15 μg produced a weak decrease in blood pressure at 18—22° C but had negligible effects at 10—12° C whereas 5-hydroxytryptamine (0.5—5 μg) produced a strong blood pressure increase at both high and low

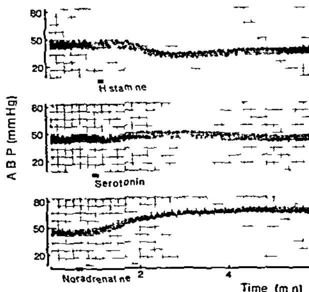


Fig 6 Blood pressure responses to histamine (? µg) 5 hydroxytryptamine (serotonin 10 µg) and noradrenaline (5 µg) in the European common viper (*Vipera berus*) The drugs were administered intravenously

temperatures (Fig 7) In perfused preparations a moderate increase in vascular resistance was often observed after the first few injections of histamine (5—15 µg) but later during perfusion experiments the effects of histamine were slight (Fig 8) A strong increase in vascular resistance after injection of 5 hydroxytryptamine (1—10 µg) was constantly present and as shown in Fig 8 this response could be abolished by adding methysergide (2 µg/ml) to the perfusion fluid Compound 48/80 (40—50 µg) had only weak effects *in vivo* Perfused preparations responded to similar doses of compound 48/80 by a long lasting increase in resistance Adrenaline and noradrenaline (5—10 µg) raised the arterial blood pressure and produced increased

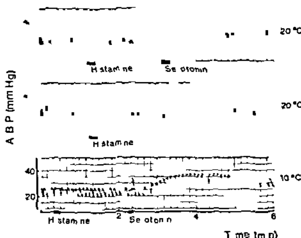


Fig 7 The two upper tracings show the effects from successive intravenous injections of histamine (1 µg) 5-hydroxytryptamine (serotonin 2 µg) and histamine (5 µg) on the arterial blood pressure of the spectacled caiman (*Caiman crocodilus*) at 20 C while the bottom tracing shows the effects of intra arterially administered histamine 5 µg and 5 hydroxytryptamine serotonin 2 µg) at 10 C

TABLE II Tissue levels of histamine (µg/g, for blood µg/ml) in reptiles*

Tissue	*Testudo hermanni*	*Emys orbicularis*	*Clemmys caspica leprosa*
Whole blood	0 05—0 15		1 2—4 1
Spleen	0 2—1 2		9 7—48 9
Liver	0 1—0 5	2 6—12 3	1 5—5 7
Lung	0 05—0 4		1 3—4 3
Whole stomach	9 4—19 5	13 0—25 5	12 8—20 6
Intestine	0 05—0 7	2 2—5 4	0 5—1 2
Esophagus	0 1—0 6		4 2—7 8
Peritoneum	0 05—0 8	1 2—10 5	0 4—4 5
Neck skin	0 01—1 0		0 9—3 6
Muscle	0 05—0 6		0 8—2 0
Tongue			

* In each species, levels show range of values obtained by assay of tissues from 4—9 animals, killed

European green lizard, the European common viper and the spectacled caiman and in skin, lung, blood and tissues from the alimentary canal of the Greek tortoise and the Spanish terrapin The tissues from the alimentary canal were the only samples which showed measurable quantities of this amine Levels within the range of 0 2—3 5 µg/g were encountered in both intestine stomach and esophagus

Histological observations

Observations on tissue mast cells and blood basophils were made in mesentery peritoneum subcutaneous tissue and blood (for terminology, cf Michels 1923) Very few mast cells were found in tissues from specimens of the Greek tortoise throughout the year even those in excellent nutritional condition and blood basophils were also scarce Similarly tissues from the Iberian land tortoise were almost devoid of

TABLE III Variations in histamine content of some tissues from the European pond terrapin, *Emys orbicularis* (six animals, numbered and listed according to the levels found in whole blood)

Animal No	Histamine content (µg/g, for blood µg/ml)					
	Whole blood	Spleen	Lung	Esophagus	Muscle	Skin
1	11 4	136 8	9 8	10 6	3 6	4 7
2	10 5	115 3	8 7	7 6	2 7	5 1
3	9 3	94 7	6 1	5 4	3 0	3 8
4	8 9	120 2	5 3	7 9	2 4	3 4
5	5 5	63 9	6 0	5 2	1 7	3 6
6	3 6	24 3	2 8	2 5	0 9	1 4

Pseudemys scripta	*Lacerta vir d s*	*V pera berus*	*Natrix natrix*	*Ca man crocod lus*
15 4—40 8		0 1—0 8	0 3—1 6	0 6—1 3
108 5—290 2				35 1—54 6
8 8—18 6	0 7—3 3	0 5—1 6	1 6—5 3	2 9—5 1
9 9—24 6	*1 6—4 0*	*0 6—2 0*	*1 2—8 5*	*10 5—17 7*
18 5—50 9	14 3—40 4		19 5—32 2	40 6—81 0
12 0—42 3	1 1—6 4	0 2—1 1	0 7—3 7	39 4—71 2
16 5—36 1	10 3—15 0	0 3—2 8	2 2—10 1	24 5—37 6
2 3—5 8	6 9—19 4		1 0—1 9	12 9—31 0
2 4—4 1				5 6—8 7
	12 5—25 6	3 1—5 8	2 5—8 4	17 5—29 8

at d fferent seasons and in varying nutrit onal states

these cells Most of the few tissue mast cells eventually encountered in the tortoises were located in subcutaneous connective tissue Unlike the tortoises the terrapins normally showed numerous tissue mast cells and also had a very high number of basophils in the blood The species richest in blood basophils was the American pond terrapin (Fig 9) However, tissue samples from mesentery and peritoneum—which in well fed terrapins were densely packed with mast cells or blood basophils at different stages of hypertrophy—showed far lower numbers of both cell types after prolonged periods of fasting Basophils in terrapin blood also showed a decrease after fasting

There was a clear species difference among terrapins both with respect to resistance of the mast cells to watery stain (toluidine blue in physiological solution) and as far as degree of degranulation induced during the preparation procedure was concerned Mast cells of the American pond terrapin were fairly resistant whereas in the Spanish terrapin many mast cells also showed degranulation after staining with alcoholic thionin even when the utmost care was taken to avoid this process Within a particular species of terrapin the mast cells (basophils) of the blood were generally more resistant than mast cells outside the blood stream but in the vicinity of small blood vessels (recently emigrated hypertrophying blood basophils) These again were more resistant than mast cells distant from blood vessels (older hypertrophied blood basophils and perhaps also histogenous forms of mast cells) The degree of metachromasia often showed similar differences being particularly pronounced in mast cells located at some distance from blood vessels

In lizards and snakes blood basophils appeared to be less abundant and less densely packed with granules than in terrapins Tissue mast cells were usually distributed throughout the connective tissues However in the European common



Fig 9



Fig 10

Fig 9 Blood smear from *Pseudemys scripta*, stained with alcoholic thionin Note the dens blood basophils which appear dark in the photomicrograph Magnification 140×

Fig 10 Mast cells in subcutaneous connective tissue of *Caiman crocodilus* as viewed thr the fluorescence microscope after treatment with o phthalaldehyde A bright and distinct ye fluorescence was initially present but the fluorescing material soon diffused out from the cells and adhered to small blood vessels and nerves Magnification 56×

vipers kept in the laboratory without food during the summer, mast cells seemed most totally absent Most of the tissue mast cells of the European green lizard a the European water snake underwent partial degranulation during preparation tissues for microscopy By using aqueous toluidine blue for staining, it was fou that the granules of these cells were highly soluble in water

Tissues from the caiman showed considerable numbers of mast cells Moreov the granulation of the mast cells of the caiman was very dense Blood basoph were not particularly numerous in this species

From observations made in species where both mast cells and tissue levels histamine were studied in the same specimens, it became clear that changes in t number of mast cells were parallelled by changes in histamine content But it w noticed that compared with histamine levels found in blood, the levels found other tissues—especially in those from the Spanish terrapin—were apparently low than would be expected if the amount of histamine in the tissue mast cell equall that in the blood basophil That the mast cell and the blood basophil actually a the main sites for storage of histamine in reptilian tissues (except the stomach) wa histochemically demonstrated by fluorescence microscopy of blood and mesentery o subcutaneous connective tissue from all the three studied species of terrapin th European green lizard the European water snake and the spectacled caiman (Fig 10) Immediately after treatment with o phthalaldehyde the fluorescing cell (which were shown by subsequent staining with alcoholic thionin to be mast cell or blood basophils) appeared bright yellow During the observation period however their fluorescence gradually faded as the fluorescing material was diffusing from the mast cells In connective tissue spreads this diffusible material seemed to adhere to small blood vessels and nerves

Discussion

Evaluation of present results Histamine has a specific stimulatory (constrictive) action on vascular smooth muscles in all studied species of turtle as indicated by the results This action which is powerful in the terrapins is only weak in the tortoises The inhibitory (dilatatory) action of histamine seems strong both in terrapins and in tortoises However due to the fact that stimulatory and inhibitory actions may balance each other the inhibitory actions were difficult to discern during *in vivo* experiments in terrapins Augmentation of the depressive effect of histamine on the arterial blood pressure of the Spanish terrapin after administration of antihistaminics is not surprising when compared to a previous observation in the rabbit (Staub 1939) Like the terrapins—but unlike most mammals—the rabbit has a strong stimulatory component in its vascular smooth muscle response to histamine (Feldberg and Schild 1930), and while stimulatory actions of histamine are easily blocked with antihistaminics inhibitory actions often persist (Goodman and Gilman 1965)

The actions of 5 hydroxytryptamine on vascular smooth muscles of turtles apparently also comprise both a stimulatory and an inhibitory component although the inhibitory component which caused a marked decrease in arterial blood pressure in all studied species is dominating

In lizards and snakes strong inhibitory actions of histamine seem to be common whereas the stimulatory actions are feeble This is consistent with absence of effects in perfused preparations and in specimens studied at low temperatures Under both these conditions, vascular tone is probably low, and the importance of such tone in revealing the inhibitory actions of histamine on vascular smooth muscle was pointed out by Dale and Richards (1918—19) Inhibitory actions of 5 hydroxytryptamine are apparently also present in lizards and snakes even though these actions may be absent in species like the European common viper of the present study A supplementary perfusion experiment in the dark green snake (*Coluber viridiflavus*) revealed that in the latter species the stimulatory actions of 5 hydroxytryptamine which were negligible in the lizards and snakes originally studied are also rather strong

Experiments performed in caimans suggest the presence of both stimulatory and inhibitory actions of histamine but the sensitivity of the vascular system to this amine is moderate Towards 5 hydroxytryptamine however, vascular smooth muscles of the caiman evidently respond by powerful contraction Inhibitory actions of 5 hydroxytryptamine were not revealed in this species

All intact animals and preparations studied—even those which failed to respond to histamine and 5 hydroxytryptamine—showed powerful responses to one or more of the drugs used for comparison (adrenaline noradrenaline, isoproterenol and acetylcholine) Absence of response to histamine and 5 hydroxytryptamine when occurring was therefore probably not due to deficiencies in the experimental procedure

Previous data on the actions of histamine and 5 hydroxytryptamine on reptilian vascular smooth muscles are sparse but those reported are consistent with the

present observations Thus Sumbal (1924), studying the tortoise *Testudo graeca* presented evidence for a dilatatory action of histamine, whereas in a recent publication Kirby and Burnstock (1969) reported that both histamine and 5 hydroxytryptamine failed to evoke any response in isolated artery strips from a lizard (*Tiliqua rugosa*) In extravascular smooth muscles, histamine has been shown to cause either contraction or relaxation of various preparations from turtles (Carlson and Luckhardt 1921) and weak and transient contraction of preparations from lizard urinary bladder (Burnstock and Wood 1967)

With respect to storage of histamine in reptilian tissues it is evident from the present results that the main histamine stores, except those of the digestive tract (non mast cell histamine in gastric mucosa cf Reite 1969 c) are the tissue mast cells and the blood basophils Large and characteristic species differences are present The nutritional state of the animal seems to be of importance in determining the individual variations in mast cell number and tissue histamine levels In reptiles living in temperate regions with long periods of dormancy this may appear as seasonal changes with the lowest number of mast cells and the lowest tissue levels of histamine occurring during spring or early summer No similar changes in tissue histamine levels or mast cell number have been found in any mammal A combination of species differences and individual nutritional dependent variations probably explains why previous observations indicated that reptilian tissues contain low levels of histamine (Wright and Trethewie 1956 Reite 1965)

The vascular responses evoked by compound 48/80 and polymyxin B indicate that both these agents may act indirectly through released mast cell histamine Moreover the histamine releasing effect of compound 48/80 was directly demonstrated *in vitro* Nevertheless it is evident that compound 48/80 and polymyxin B have marked effects other than those mediated by released histamine Apart from their vascular actions histamine and 5 hydroxytryptamine themselves apparently also produce numerous additional effects

Significance of present results compared to those obtained in other vertebrates The present work provides ample evidence that vascular smooth muscles of reptiles show both inhibitory and stimulatory components in their responses to histamine and 5 hydroxytryptamine These response patterns which are characteristic of the mammalian vascular system (cf Reite 1969 a) seem to have appeared rather late in the evolutionary history of vertebrates By summarizing information contained both in previous reports (Reite 1969 a b and in this paper the evolution of vascular smooth muscle responses to histamine and 5 hydroxytryptamine (compared to the evolution of responses to adrenaline noradrenaline and acetylcholine) may be presented schematically as shown in Table IV

To ascertain that the African lungfish which with its powerful vascular response to histamine was unique among the studied species of fish (Reite 1969 a), is not unique among lungfish as well I obtained supplementary information in the Australian species *Neoceratodus forsteri* The blood pressure effects of histamine in the Australian lungfish depicted in Fig 11 resemble those previously demonstrated

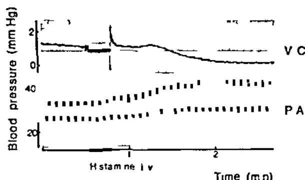


Fig 11 Effects of intravenously administered histamine (50 µg) on the blood pressure in the pulmonary artery (P A) and the vena cava (V C) of the Australian lungfish *Neoceratodus forsteri* (body weight ca 6 kg water temperature ca 20° C)

in its African relative Thus strong vascular responses to histamine have apparently evolved at least twice in the immediate ancestors of lungfish and in the immediate ancestors of primitive reptiles Evolution of the mast cell as storage site for histamine *seems to parallel the evolution of strong vascular actions of histamine whereas the* presence of non mast cell histamine stores in the digestive tract is linked with the presence of gastric acid secretion (Reite 1968 1969 c)

Amphibians constitute the only class of vertebrates in which all studied species show a fairly uniform vascular response to 5 hydroxytryptamine (vasodilatation Reite 1969 b) and most amphibians are also characterized by the presence of large quantities of this amine in their skin (Erspamer 1961) The vasoconstrictor effect of 5 hydroxytryptamine on branchial blood vessels in bony fish (Reite 1969 a) may reflect a functional specialization

In comparison with adrenaline noradrenaline and acetylcholine which produce principally similar actions on vascular smooth muscles from all vertebrates and for which definite roles as hormones or neurohumors have been revealed the effects of histamine and 5 hydroxytryptamine are highly varied (cf Table IV) Possible roles for the latter amines in the physiological control of vascular smooth muscle should therefore be sought within limited groups of vertebrates The fact that inhibitory components in the vascular actions of histamine and 5 hydroxytryptamine have only been demonstrated in vertebrates in which the blood vessels are under sympathetic nervous influence may be a guide to the mechanisms involved in this mode of action Another fact which deserves mention is that both storage of histamine in mast cells and vascular responses to histamine apparently evolved in animals subjected to considerable environmental changes spending part of their time in water and part of their time in air Further work including studies on formation and inactivation of histamine and 5 hydroxytryptamine in lower vertebrates seems required It may be found that despite conspicuous vascular actions the extravascular actions of these amines are physiologically the more important

Concluding remarks Presence of pharmacological responses to a substance and occurrence of this substance in the tissues may be looked upon as separate characteristics which may appear independent of each other before acquiring functio

TABLE IV A schematic representation of the distribution of specific actions of histamine and 5-hydroxytryptamine on vascular smooth muscle in vertebrates (actions of some other pharmacological agents are included for comparison) *

Pharmacological agent	Type of action (stimulatory +, inhibitory —, dual ±, actions absent or negligible 0)				
	Jawless vertebrates	Fish	Amphibians	Reptiles	Birds and mammals
Histamine	0	0 (+ in lung fish)	0	±	±
5-hydroxytryptamine	0	+	±	±	±
Acetylcholine	+	+	±	±	±
Adrenaline	±	±	±	±	±
Noradrenaline	±	±	+	±	±
Isoproterenol	—	—	—	—	—

* No attempt is made here to consider separately vascular beds which may be functionally specialized

significance and also be retained after the functional significance is lost. Accordingly the vascular actions of histamine and 5-hydroxytryptamine in mammals may reflect that these substances have a function in this class of vertebrates, but it may just as well reflect that they once had a function in mammalian ancestors or are candidates for a function in mammalian descendants of the future. However, the evolution of a set of pharmacological characteristics in an animal would probably not take place if these characteristics did not have selective value by being useful in the interaction of that animal with its physical and biological environment. Disappearance of characteristics which are no longer of any use but remain harmless will on the other hand be by chance alone and large species differences must be expected to occur.

The series of studies on the evolution of vascular smooth muscle responses to histamine and 5-hydroxytryptamine which is completed with the present work does not offer any solution as to the functional significance of these amines in relation to vertebrate vascular smooth muscle. My investigations have, however, produced a background which may prove helpful in deciding where to search. Considering the above comments, studies in lungfish and reptiles may be most rewarding in the elucidation of possible vascular functions of histamine, whereas amphibians appear to be more suitable for studying the role of 5-hydroxytryptamine.

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Acta physiol scand 1970 78 232—235

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Effect of the C-Terminal Octapeptide of Cholecystokinin on Guinea Pig Ileum and Gall-Bladder in vitro

By

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Abstract

HEDNER, P *Effect of the C terminal octapeptide of cholecystokinin on guinea pig ileum and gall bladder in vitro* Acta physiol. scand 1970 78 232—235

The C-terminal octapeptide of cholecystokinin shares the pharmacological properties of the original molecule Both seem to act directly on the smooth muscle cell of the gall bladder but indirectly via a nervous pathway in the ileum. Both reduce the resistance of the choledocho-duodenal junction. This pharmacological profile is characteristic for a group of substances which besides cholecystokinin and its C-terminal octapeptide also includes gastrin pentagastrin and caerulein. There is a close chemical relationship between these substances *e g* they all have the same C-terminal tetrapeptide

Effective doses for the octapeptide were 0 1—0 5 ng/ml *in vitro* Its molar activity in the gall bladder and ileum is comparable to that of caerulein and about 3 times that of cholecystokinin and 1000 times that of pentagastrin

Besides the heterologous caerulein the C-terminal octapeptide of cholecystokinin appears as the most active gall bladder contracting agent known at present with a molar activity about 100 times that of acetylcholine histamine or bradykinin

In 1964 Jorpes *et al* reported a purification procedure for extracted cholecystokinin yielding a product with an activity of about 3000 Ivy dog units per mg This product was pure enough to permit a structural analysis The hormone is a linear polypeptide with 33 amino acids where the amino acid sequence has been elucidated by Mutt and Jorpes 1968 They observed that among the products resulting from degradation with trypsin the C terminal octapeptide contained the characteristic effects of cholecystokinin pancreozymin on gall bladder and pancreas This C-terminal octapeptide with the amino acid sequence Asp-Tyr(SO_3 Met Gly Trp Met Asp-PheNH₂ has been synthetized by Dr M A. Ondetti New Brunswick N J USA Rubin and Engel 1968 reported its contractile activity on the guinea pig's gall bladder to be about 30 000 Ivy dog units per mg in vivo and 0 3—2.5 ng/ml was active in vitro

Extracted cholecystokinin has been shown to contract the guinea pig's gall bladder by an effect directly on the smooth muscle cell but to contract the small intestine by another mechanism of action. namely via a cholinergic nervous pathway without

cholinergic synapses (Hedner and Rorsman 1968) This study was undertaken to investigate if these different effects of extracted cholecystokinin were bound to the same part of the molecule, namely to its C-terminal octapeptide

Material and methods

Guinea pigs of either sex weighing 200—300 g were used

The in vitro experimental equipment and technique have been described earlier (Hedner and Rorsman 1968) In the investigation of the choledochoduodenal junction in the cat the experimental procedure was essentially the same as described earlier (Hedner and Rorsman 1969)

was obtained from Prof E Jorpes of cholecystokinin was supplied by agastrin (Peptavlon) by ICI/Scan

Results

1 Gall bladder The octapeptide regularly elicited a submaximal contraction of the gall bladder in a concentration of 0 5 ng/ml which is in accordance with the results reported by Rubin and Engel (1968) Comparable responses were obtained by cholecystokinin, 0 015 units/ml, and by pentagastrin 1 μg/ml These responses were not affected by atropine in a dose (0 08 μg/ml) that completely abolished the corresponding response to acetylcholine The responses were also resistant to tetrodotoxin 0 4 μg/ml

2 Ileum, longitudinal muscle layer When assayed against extracted cholecystokinin on this preparation the potency of the octapeptide was found to be 36 000 units/mg The lowest regularly effective dose was 0 5 ng/ml The ileum also responded to pentagastrin the response to 2 μg/ml roughly being equivalent to that given by 1 ng/ml of the octapeptide Both responses were reduced slightly (by about 20 %) or not at all by hexamethonium 16 μg/ml Both responses were completely inhibited by tetrodotoxin 0 4 μg/ml which left the response to acetylcholine 0 02 μg/ml unaffected After this dose of tetrodotoxin the response to acetylcholine was not increased in the presence of cholecystokinin or the octapeptide

Atropine 0 08 μg/ml completely blocked the responses to acetylcholine cholecystokinin pentagastrin and to the octapeptide the dose of the latter being increased even by 2 log units without appearance of a response

3 Ileum circular muscle layer In the experiments reported here the response of the circular muscle layer consisted of a propagated contraction Such a response could be obtained by increasing the intraluminal volume, by administration of acetylcholine (0 01 μg/ml) probably as a reflex elicited via contraction of the longitudinal muscle layer (Hedner and Rorsman 1968) by the octapeptide (down to 0 1 ng/ml) and by cholecystokinin (down to 0 003 units/ml) Hexamethonium 8 μg/ml inhibited the response to increased intraluminal volume to acetylcholine and sometimes to the lowest effective doses of the octapeptide and cholecystokinin but not to higher doses (over 1 0 ng/ml and 0 03 units/ml respectively) Tetrodotoxin 0 1 μg/ml inhibited the responses to increased intraluminal volume and to acetylcholine and to the octapeptide and cholecystokinin as well

4 Choledochoduodenal junction (cat) In a few experiments on the anesthetized cat the octapeptide regularly reduced the flow resistance of the choledochoduodenal junction The effect of 30 ng/kg of the octapeptide i v roughly equalled that of 1 Ivy dog unit/kg of extracted cholecystokinin [1]

Discussion

These experiments indicate that the C terminal octapeptide of cholecystokinin shares the pharmacological characteristics of the original molecule Thus both seem to have a direct effect on the smooth muscle cell of the gall bladder but in the ileum an effect mediated via a nervous pathway which is cholinergic at least in the longitudinal layer For the circular layer another hitherto unknown transmitter may be actual (Kottegoda 1969) Both substances reduce the resistance of the choledochoduodenal junction

Among all the substances able to elicit contractions of the smooth muscle in both gall bladder and small intestine one can now recognize a group with the characteristic pharmacological profile outlined above This group includes cholecystokinin (Naito *et al* 1963 Hedner *et al* 1967) gastrin (Bennett 1965) caerulein (Bertaccini *et al* 1968 the C-terminal octapeptide of cholecystokinin and pentagastrin A chemical relationship corresponds to the pharmacological one Porcine cholecystokinin has the amino acid sequence Lys(Ala Gly Pro Ser)Arg Val(Ile Met Ser) Lys-Asn(Asx Glx His Leu Pro-Ser)Arg Ile(Asp-Ser)Arg Asp(Gly Met-Try Tyr (SO_3)) Asp-PheNH_2 (Mutt and Jorpes 1968a) porcine gastrin II is Pyr Gly Pro-Try Met Glu Glu Glu Glu Glu Ala Tyr(SO_3)Gly Try Met Asp PheNH_2 (Gregory *et al* 1964 caerulein is Pyr Gln Asp-Tyr(SO_3) Thr Gly Try Met Asp-PheNH_2 Anastasi *et al* 1967) and the C terminal octapeptide of cholecystokinin has the amino acid sequence Asp Tyr(SO_3) Met Gly Try Met Asp-PheNH_2 (Mutt and Jorpes 1968b Pentagastrin is the C terminal tetrapeptide of gastrin elongated at the amino end by a t butyloxycarbonyl β alanyl residue Thus all substances have the C-terminal pentapeptide in common with the exception of pentagastrin in which the similarity is limited to the tetrapeptide Caerulein has the same C terminal octapeptide as cholecystokinin with the exception of a threonine instead of the second methionine counted from the C terminal end

Compared with extracted cholecystokinin the C terminal octapeptide is about 10 times more active on a weight basis and about 3 times more active on a molar basis Regarding the activity the only substance that warrants a comparison with the octapeptide is caerulein In the isolated guinea pig gall bladder effective doses for caerulein have been reported to be 0 5—2 ng/ml (Bertaccini *et al* 1968) for the C-terminal octapeptide of cholecystokinin to 0 3—2 5 ng/ml (Rubin and Engel 1968) which compares well with the effective doses for the octapeptide found in this investigation Effective doses for pentagastrin were about 1 μg/ml In the guinea pig ileum in vitro (longitudinal layer) the effective doses of these substances are as a rule

[1] These experiments were performed by Dr G Liedberg Dept of Surgery Lund Sweden

slightly higher than in the gall bladder perhaps more pronounced for caerulein of which 15—20 times higher doses are required in the ileum than in the gall bladder (Bertaccini *et al* 1968)

Thus the increase from the C-terminal four amino acid sequence to the C-terminal octapeptide of cholecystokinin leads to about a 1000 fold increase in activity both in the gall bladder and in the ileum The substitution of the first methionine in this octapeptide by threonine and the addition of proline and glutamine at the N terminal end resulting in caerulein does not lead to any noteworthy change of activity in the gall bladder but possibly to a certain reduction of activity in the ileum

Caerulein has not been found in the mammalian body but still seems to have the same high activity as the autologous octapeptide As they are only slightly different structurally it is possible that they act on the same receptor but this still remains to be proved

Besides the heterologous caerulein the C-terminal octapeptide of cholecystokinin appears as the most active gall bladder contracting agent known at present, with a molar activity about 100 times that of acetylcholine histamine or bradykinin All the different effects of cholecystokinin on gall bladder choledochoduodenal junction small intestine and pancreas seem to be contained in its C-terminal octapeptide

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The Metabolism of Nicotine and Cotinine by a Mouse Liver Preparation

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Abstract

STÅLHANDSKE T *The metabolism of nicotine and cotinine by a mouse liver preparation* Acta physiol scand 1970 78 236—248

The metabol ... of mouse liver
homogenate ... sm of nicotine
was found to ... the formation
of cotinine γ ... esented Three
unidentified ... lites were also
excreted in urine after intraperitoneal administration of nicotine Chromatographical evidence revealed that cotinine is metabolized to hydroxycotinine, γ (3 pyridyl)-γ oxo-N methylbutyramide and/or demethylcotinine and one unidentified metabolite No formation of $^{14}CO_2$ was observed

Studies on the metabolism of nicotine in the mouse have established the liver as the principal organ of detoxication (Hansson *et al* 1964) In experiments with liver slices they presented chromatographical evidence for a formation of the nicotine metabolites cotinine γ-(3 pyridyl) γ-oxo N methylbutyramide, hydroxycotinine CO_2, and two *unidentified metabolites Cotinine was found* to be the major metabolite

Except the investigation by Bowman *et al* (1964) no data concerning the metabolism of cotinine in the mouse have been published I v administration of cotinine resulted in a urinary excretion of hydroxycotinine and demethylcotinine Evidence for the presence of γ (3-pyridyl)-γ oxo-N methylbutyramide was also presented

The present work concerns qualitative and quantitative aspects on the metabolism of nicotine and cotinine in a 10 000×g fraction of mouse liver homogenate

The in vivo metabolism of nicotine as reflected by the excretion of urinary metabolites is also studied

Methods

Experimental animals

Male albino mice of NMRI strain weighing 20—25 g were used. Prior to use the mice were starved for 18—20 hrs.

Compounds

[illegible] 1962 [illegible]

chromatographically.

Preparation of the 10 000 × g liver supernatant

The mice were sacrificed by cervical dislocation and bled by decapitation. The liver was immediately excised and rinsed in cold 0.2 M phosphate buffer pH 7.4 and homogenized in two volumes of the same buffer at +2° C. The homogenization was carried out with a Waring blendor homogenizer and the livers of three mice were homogenized together (total volume of homogenate about 9 ml).

The homogenates were centrifuged at 10 000 × *g* in a refrigerated centrifuge at +2° C for 20 min to sediment nuclei, mitochondria and cell debris. The supernatant containing the microsomal and soluble fractions of the liver cell was then frozen to −20° C and after 20 hrs thawed and used as an enzyme source. Prior to use the supernatant was diluted with one volume of the buffer.

Protein estimation

The protein concentration of the supernatant used ranged between 20 and 25 mg/ml. The protein was determined according to the Biuret method (Szarkowska and Klingenberg 1963). Crystalline serum albumine was used as a standard.

Incubations

To 1.5 ml *supernatant fraction* kept in a 25 ml Erlenmeyer flask was added 0.5 ml of a solution containing 25 μmoles nicotinamide, 37 μmoles $MgCl_2$, 50 μmoles KCl, 0.2 μmoles TPN, 10 μmoles glucose-6-phosphate in a 0.2 M phosphate buffer pH 7.4. The concentration of all co-factors were optimal. Solutions of nicotine and cotinine in distilled water in a total volume of 0.5 ml were then added. The flasks were shaken in a Dubnoff shaker in an atmosphere of air at 37.5° C. The reaction was stopped by adding 0.7 ml N HCl. In some incubations the incubation flasks were closed in order to make it possible to collect formed $^{14}CO_2$. No differences in enzyme activity were found between closed and open flasks.

When incubations were carried out in nitrogen atmosphere the flasks were covered with a plastic hood and nitrogen was flushed through during the experiment.

One or two bottles without any co-factors were always run parallel as controls.

For substrate curves 6 different substrate concentrations ranging from 0.2—6.4 mM were used. Under the assay conditions used the enzymatic reaction was linear with time and proteins.

Nicotine used for substrate curves was a mixture of ^{14}C-nicotine and non-labelled nicotine bitartrate and the amounts of used nicotine were calculated as the pure base. To reach the concentration of 1.6 mM of cotinine used in some experiments it was necessary to use a mixture of non-labelled (−) cotinine and (±) ^{14}C-cotinine.

To evaluate K_m and V_{max} initial velocity v was plotted against v/[S] ([S] = substrate concentration) (Hofstee 1952). The function of the best fitting line was determined by the method of least squares and the values of K_m (Michaelis constant) and V_{max} (maximum velocity of the reaction) calculated from the function.

Extractions

For *quantitative determination* of cotinine and nicotine after incubation the contents of the incubation flask and 2 × 1 ml of the phosphate buffer pH 7.4 used for rinsing were transferred to a centrifuge flask and cotinine and nicotine was extracted according to the method described by Hucker *et al.* (1960). The recovery of nicotine and cotinine was found to be above 90 % and 85 % respectively.

For *chromatographic studies* the contents of the incubation flasks or collected urine were transferred to a shaking bottle The flasks were rinsed with 1 0 ml water Enough 0 1 N NH_4OH was added to adjust pH to 9 0 The mixture was extracted by shaking with 10 ml chloroform methanol (2 1) (Hansson *et al* 1964) and the two phases were separated by centrifugation This was repeated twice and the phases from each extraction were pooled

The radioactivity obtained in the different phases by the methods used were determined in a Packard Tri Carb liquid scintillation counter after addition of ethanol and 1 % PPO in toluene

Collection of carbon dioxide

To collect $^{14}CO_2$ a filter paper soaked with 5 N NaOH was placed above the incubation mixtu [illegible]
ten r [illegible]
into [illegible]
2 5 I [illegible]
Th [illegible]
$Ba^{14}CO_3$ when acid was added

Chromatography

The chloroform and methanol water phases were reduced in volume under N_2 and spotted on 250 μ thick silica thin layer plates for chromatography Thin layer chromatography (Stahl 1962) on silica gel (kieselgel water w/v 30 60) was used Plates were activated by heat for 2 hrs *The solvent system for thin layer chromatography found to give the most complete* [illegible]

N methylbutyramide in this system but presented no problems in the case of nicotine [illegible]
since the ^{14} [illegible] a spot on
the autora [illegible] cotinine
metabolism [illegible] ed are both
radioactive [illegible] tinine "Y"
and Y a [illegible] Three other
solvent systems were used to check the results namely phenol water (80 30 w/v) (solvent B) chloroform methanol (85 15 v/v) and acetone ethanol (80 0 v/v) Development time for the chromatograms was between 20 and 30 minutes with the exception of the last system which required about 2 1/2 hrs The solvent front ran straighter and the development times were shorter when the walls of the chromatography chamber were covered with filter paper soaked in the solvent In all cases the front was allowed to run for approximately 12 5 cm. Non radioactive standard compounds were located by spraying the plates with p amino benzoic acid in 96 % ethanol (1 % w/v) and exposing the plates to cyanogen bromide vapour

After drying the plate in the air radioactive compounds were located by exposing the plate to Kodak No screen X ray film The exposure times varied but never exceeded six weeks

Viewing by means of an ordinary X ray film viewer of the autoradiograms upon which the thin layer plates were superimposed allowed the radioactive spots to be marked These areas were then scraped off with a razor blade into 20 ml liquid scintillation vials and counted as a suspension in 10 ml 0 5 % 2 5 DPO + 0 03 % dimethyl POPOP + 4 % silica gel (Aerosil) in toluene (Snyder and Stephens 1962) When quantitations from chromatograms were made controls with radioactive nicotine and cotinine were always run parallel on chromatograms and radioactive spots caused by auto-oxidative products were measured When auto-oxidative products observed and metabolites had corresponding R_f values the amount was always taken into account when the amount of metabolite was calculated About 90 % of the activity put on chromatographical plates was usually recovered after scraping

Collection of urine

The animals weighing about 25 g were injected i v with 2 5 mg ^{14}C-nicotine per kg b w and during urine collection kept in metabolic cages for 4 hrs

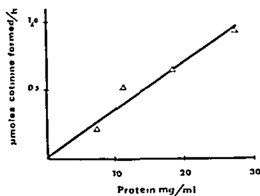


Fig 1

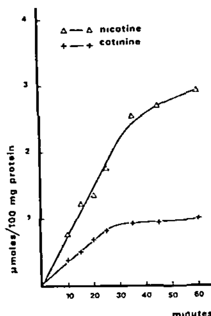


Fig 2

Fig 1 Rate of cotinine formation with varying concentrations of supernatant protein Each value is a mean from three incubations Nicotine 1 6 mM was incubated with co-factors described under methods

Fig 2 Rate of metabolism of nicotine and formation of cotinine Each value is a mean from three incubations with portions of a supernatant fraction prepared from livers of 15 mice Nicotine 1 6 mM was incubated with co-factors described under methods

Results

Metabolism of nicotine

I Kinetic studies

Studying the metabolism of nicotine both the disappearance of nicotine and the appearance of formed cotinine were estimated The total metabolism of nicotine and the formation of cotinine measured under the conditions described above were found to be proportional to the amount of protein added (Fig 1) and linear with time up to at least 30 and 25 min of incubation respectively (Fig 2)

Fig 3 shows the rates of nicotine disappearance and cotinine formation as a function of substrate concentration The optimal reaction velocity was approached at a nicotine concentration of 1 6 mM

The apparent K_m value obtained for the rate of disappearance of nicotine was found to be lower than the K_m value calculated for the formation of cotinine ($p < 0\ 05$)

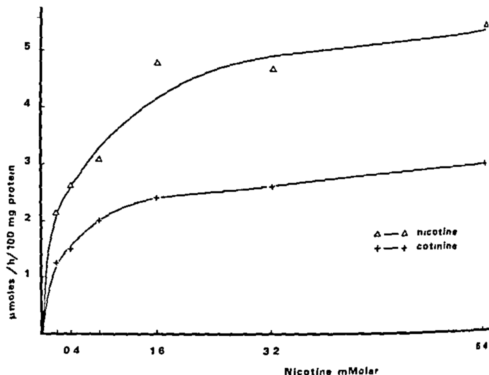


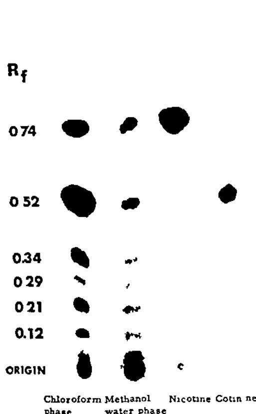
Fig 3 Relationship between nicotine concentrations and rate of nicotine metabolism and cotinine formation Each value is a mean from three incubations with portions of a supernatant fraction prepared from livers of 15 mice Nicotine was incubated for 20 minutes with co-factors described under methods
Nicotine metabolism $V_{max} = 4\ 8$ µmoles/h/100 mg protein, $K_m = 0\ 23$ (mM)
Cotinine formation $V_{max} = 2\ 9$ µmoles/h/100 mg protein $K_m = 0\ 30$ (mM)

II *Chromatographical studies*

Extraction of the incubation mixture with chloroform methanol (2 1 v/v) after incubation of nicotine with the 10 000×g fraction of mouse liver homogenate revealed that more than 80 per cent of the recovered radioactivity (85 % recovery of added radioactivity) was present in the chloroform phase Autoradiograms of chromatograms run in solvent A revealed that the chloroform and the methanol water phases contained the same number of compounds with the same R_f value (Fig 4)

The radiochromatogram showed five separate radioactive metabolites which since the ^{14}C-label is in the methylgroup do not include demethylated compounds Three of the spots were found to have the same R_f values as the reference substances cotinine ($R_f = 0\ 52$) γ (3 pyridyl) γ oxo-N methylbutyramide ($R_f = 0\ 34$) and hydroxycotinine ($R_f = 0\ 21$) The remaining two spots X' ($R_f = 0\ 29$) and Y'' ($R_f = 0\ 12$) did not co chromatograph with any of the available references

Despite rigorous attempts to standardize the chromatographic methods there occurred deviations of the R_f values from plate to plate The relative positions of various metabolites were however, always constant



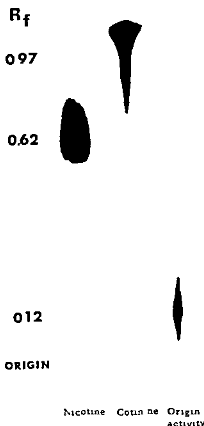


Fig 4

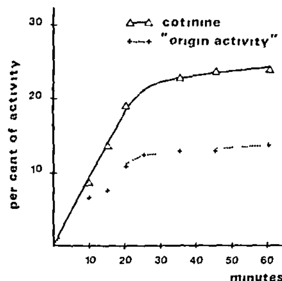
Fig 5

Fig 4 Autoradiogram of thin layer chromatogram showing nicotine metabolites extracted in the chloroform and methanol water phases after incubation of 0 4 mM ^{14}C-nicotine for 25 minutes Solvent system ethanol acetone benzene conc NH_4OH (5 40 50 5 v/v) Radioactive components and their ratio in percent of total radioactivity on the chromatogram nicotine (R_f=0 74) 3 6 % cotinine (R_f=0 52) 77 8 % γ (3 pyridyl)-γ oxo-N methyl butyramide (R_f=0 34) 1 2 % "X" (R_f=0 29) 0 2 % hydroxycotinine (R_f=0 21) 0 7 % "Y" (R_f=0 12) 0 2 % origin activity 15 7 %

Fig 5 Autoradiogram of phenol water (80 30 w/v) activity (R_f=0 12) The of chromatograms obtained of ^{14}C nicotine are run in the solvent ethanol-acetone benzene-conc NH_4OH (5 40 50 5 v/v) (Fig 4)

After chromatography of both the chloroform and the methanol water phases some radioactivity always remained in the origins of the chromatograms (origin activity) (Fig 4) The activity present in origin was responsible for about 10 per cent of the total activity in the chloroform phase and for as much as about 80 per cent of this activity in the methanol water phase

To get further information about the identity of the radioactive compound(s)



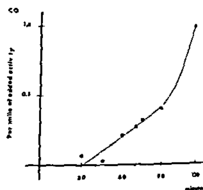


Fig 6

Fig 7

Fig 6 Rate of cotinine (Δ———Δ) and "origin activity" (+ - +) formation The values represent quantitative data obtained from chromatograms of chloroform phases after incubations of nicotine and are expressed as the percentage of total radioactivity on the chromatogram 1 6 mM nicotine was incubated

Fig 7 Rate of CO_2 formation after incubation of 1 6 mM nicotine Each value represents a mean from at least two determinations and is expressed in per mille of added radioactivity of ^{14}C-nicotine

remaining in the origins, this area was scraped off, the compound(s) resolved and rechromatographed The origin activity' moved only in solvent B of used solvents The activity was not separated and showed a pronounced tailing None of the available reference compounds co chromatographed with this activity (Fig 5) Consequently it was not caused by any of the above mentioned metabolites or nicotine

The amount of activity found in origin on chromatograms of the chloroform phases increased with time of incubation (Fig 6) This course was in correspondence with the formation of cotinine with time (Fig 6 and 2)

Experiments were also performed in order to show the dependence of oxygen and TPNH for the metabolism of nicotine The lack of oxygen produced with a constant flushing of nitrogen throughout the experiment caused a significant decrease in the amount of formed cotinine The amount of startpoint activity was also drastically decreased under anaerobic conditions

The total amount of minor metabolites was also decreased but the decrease was smaller (Table I)

In incubations performed in the absence of added TPN and glucose-6-phosphate there was no metabolism of nicotine β diethylaminoethyl diphenylpropylacetate (SKF 525-A) (0 5 10^{5} M), a compound that suppresses the metabolic transformation of many drugs (Cooper *et al* 1954) also inhibited the metabolism of nicotine (Table I)

TABLE I Effect of SKF 525 A lack of TPNH generating system and oxygen on the metabolism of nicotine Distribution of radioactivity between nicotine and its metabolites after incubations with 1 6 mM nicotine for 25 min The compounds were extracted in the chloroform phase and identified chromatographically Each value represents a mean of 3 expts

Incubation conditions	Percentage of total radioactivity on chromatograms			
	Nicotine	Cotinine	Minor metabolites	Origin
Standard conditions*	76 4	12 9	1 2	9 9
In atmosphere of nitrogen	94 0	3 8	1 0	1 2
No TPN and glucose 6 phosphate	99 8	—	—	—
Standard conditions* and addition of 0 5 10⁻³ M SKF 525-A	87 9	7 5	1 0	3 6

* Conditions see methods

The formation of cotinine quantitatively determined by extraction after incubation confirmed the above mentioned results (Table II)

III Formation of ^{14}C carbon dioxide

The formation of carbon dioxide was measured in order to show if any carbon dioxide as a result of demethylating activity, is formed from nicotine It was not possible to detect any release of carbon dioxide in incubations shorter than 30 min After that time there were rapidly increasing amounts of carbon dioxide formed However, the amounts of carbondioxide recovered even after 2 hrs of incubation were small After 2 hrs of incubation the amounts of collected carbon dioxide were equivalent to 0 04 μmoles of demethylated nicotine (Fig 7)

TABLE II Effect of SKF 525 A lack of TPNH generating system and oxygen on the formation of cotinine Cotinine formation after incubation with 1 6 mM nicotine for 25 min Results are mean values of at least 3 determinations

Incubation conditions	μmoles cotinine/hr/100 mg protein
Standard conditions*	2 33
In atmosphere of nitrogen	0 23
No TPN and glucose-6-phosphate	—
Standard conditions* and addition of 0 5 10⁻³ M SKF 525-A	1.24

* Conditions see methods

TABLE III Distribution of radioactivity between nicotine, cotinine and their metabolites formed after incubation with 1 6 mM nicotine and 1 6 mM cotinine Incubation time 25 min. The compounds were extracted in the chloroform phase Nicotine and cotinine were identified chromatographically Each value represents a mean of 3 expts

	Percentage of total radioactivity on the chromatograms			
	Nicotine	Cotinine	Minor metabolites	Origin
Nicotine 1 6 mM	76 4	12 9	1.2	9 9
Cotinine 1 6 mM		95 3	4 7	—

Metabolism of cotinine

Chromatographical studies

Extraction of the incubation mixture with chloroform methanol (2 1 v/v) after incubation of cotinine with the 10,000×g fraction of mouse liver homogenate revealed that more than 97 % of the recovered activity was present in the chloroform phase Radiochromatograms of the methanol water phase showed that the activity in this phase was mainly caused by ^{14}C-cotinine Radiochromatograms for the chloroform phase in solvent A showed 3 radioactive metabolites The metabolites had R_f values corresponding to , (3 pyridyl) γ-oxo-N methylbutyramide and demethylcotinine (R_f=0 34) hydroxycotinine (R_f=0 22) and compound Y" (R_f=0 13) No activity was found in the origin (Fig 8) The mutual ratio of the metabolites indicates a very low metabolism of cotinine in the in vitro system used The recovery of added cotinine (1 6 mM) after extraction was close to 100 % after finished incubation also indicating a low metabolism of cotinine

No formation of ^{14}C carbon dioxide could be detected when cotinine was incubated under the same conditions as described for nicotine

A comparison of nicotine and cotinine metabolism

The same concentrations of nicotine and cotinine (1 6 mM) were incubated under identical conditions Radiochromatograms of the chloroform phases reveal that despite a lower disappearance of cotinine more minor metabolites were formed from cotinine than from nicotine (Table III)

Urinary metabolites

The excreted urine collected for 4 hrs contained 46 % of the radioactivity injected After extraction of the urine with chloroform methanol (2 1 v/v) 95 % of the radioactivity was recovered Of the extracted radioactivity 82 % was found in the chloroform phase The methanol water and the chloroform phases were subjected to

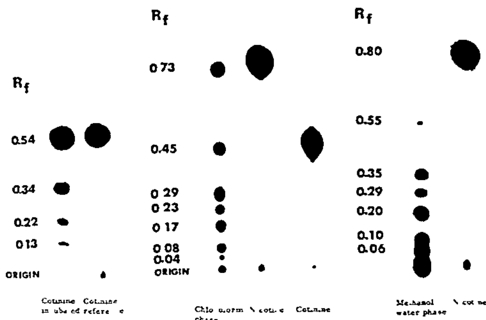


Fig 8 Fig 9

Fig 8 Autoradiogram of thin layer chromatogram showing cotinine metabolites extracted in the chloroform phase after incubation of 0 2 mM ^{14}C-cotinine for 25 min. Solvent system ethanol acetone benzene-conc NH_4OH (5 40 50 5 v/v) Radioactive components and their ratio in percent of total radioactivity on the chromatogram cotinine (R_f=0.54) 96 4 %, γ (3-pyridyl)-γ-oxo-N methylbutyramide and/or demethylcotinine (R_f=0 34) 2 5 % hydroxy cotinine (R_f=0 21) 0 6 % "Y" (R_f=0 12) 0 5 % "origin activity" 0 0 %

Fig 9 Autoradiograms of thin layer chromatograms of the chloroform and methanol water phases of urine collected for four hours after injection of 2 5 mg/kg ^{14}C-nicotine Solvent system ethanol acetone-benzene-conc NH_4OH (5 40 50 5 v/v) Radioactive components and their ratio in per cent of total radioactivity on the chromatogram nicotine (R_f=0 73 0 80*) 15 5 % cotinine R_f=0 45 0 55*) 13.3 % γ (3-pyridyl-γ-oxo-N methylbutyramide (R_f=0 29 0 35*) 15 9 % "X" (R_f=0 23 0 29*) 7 9 % hydroxycotinine (R_f=0 17 0 20*) 16 6 % "Y" (R_f=0 08 0 10*) 10 9 % "Z (R_f=0 04 0 06*) 3.3 % "origin activity" 14 8 %

* Methanol water phase.

chromatography in solvent A. 65 % of the activity in the chloroform phase and as much as 64 % of the activity in the methanol water phase did not move in the chromatographical solvent used The radiochromatograms show that all the radioactive nicotine metabolites observed in the above mentioned in vitro studies are also present as urinary metabolites Another compound called "Z with a very low R_f value (0 04) was also observed (Fig 9)

Discussion

All the nicotine metabolites formed during incubations with mouse liver slices (Hansson *et al* 1964 are also observed in incubations performed with the 10 000 × *g* supernatant fraction of mouse liver homogenate This finding indicates a localiza

tion of the nicotine metabolizing enzymes to the hepatic microsomal fraction as was suggested by Hucker *et al* (1960)

The inhibition of nicotine metabolism observed under anaerobic conditions and in supernatants not supplemented with TNP and glucose 6 phosphate necessary for generation of TPNH, is in agreement with earlier findings presented by Hucker *et al* (1960) They demonstrated the presence of TPNH and O_2 requiring nicotine metabolizing enzymes in the microsomal fraction of rabbit liver They also observed that SKF 525 A inhibits the in vitro metabolism of nicotine, which was confirmed in the present study

In accordance with the present findings the major nicotine metabolite formed in mice in vivo and in vitro is cotinine (Hansson *et al* 1964) The formed cotinine seems to be fairly stable to further metabolism in the in vitro system used in this study The metabolism of cotinine was only one fifth of that of nicotine when the same concentrations of nicotine and cotinine were incubated under identical conditions This supports the observations by Papadopoulus and Kintzios (1963) and Hucker *et al* (1960) who found that only a small fraction of added cotinine was metabolized during incubation with supernatants of rabbit liver

Radiochromatograms indicate that hydroxycotinine γ (3 pyridyl) γ oxo N methylbutyramide and/or demethylcotinine are the cotinine metabolites formed by mouse liver enzymes

Bowman *et al* (1964) presented evidences for a urinary excretion of hydroxy cotinine demethylcotinine and γ-(3 pyridyl)-γ oxo N methylbutyramide after giving mice tritiated (−) cotinine

In similar experiments with rats McKennis *et al* (1962) showed that administration of (−) cotinine led to the urinary excretion of hydroxycotinine, demethyl cotinine and γ (3 pyridyl) γ oxo N methylbutyramide

The increased amount of 'minor metabolites' obtained in incubations with cotinine as compared with incubations of nicotine also suggests that the 'minor metabolites' are formed in metabolic steps passing cotinine This includes the formation of the unidentified metabolite 'Y' but not 'X'

After incubation of nicotine the 'origin activity' was always present Its chromatographical properties in solvent B indicates that it is caused by a nicotine metabolite The formation of 'origin activity' as a function of time in incubations with nicotine further supports this suggestion and indicates that this activity is not formed in a metabolic pathway including cotinine This activity was not observed in incubations with cotinine The 'origin activity' is therefore very likely formed directly from nicotine

A comparison of the relative amounts of "origin activity" and cotinine formed at a low and at a high nicotine concentration reveals that the formation of cotinine is definitely favoured at the low substrate concentration One may postulate the presence of two different enzyme systems one forming [illegible] activity and one cotinine As a fact the kinetic constants for cotinine f[illegible] and nicotine disappearance are different As a consequence of this and [illegible] rate [illegible] nicotine

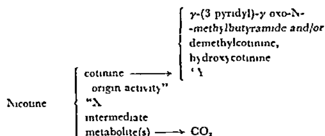


Fig 10 Schematic representation of nicotine degradation in the mouse liver in vitro

is in the mouse very likely metabolized via at least two different enzymatic pathways simultaneously One including and one not including the intermediate cotinine but another not demethylated compound This is also suggested to be the case in other animals (for review see Larson *et al* 1968)

The demethylated products of nicotine, nornicotine and demethylcotinine have been observed as metabolites of nicotine in mammals (McKennis *et al* 1962, Papadopoulus and Kintzios 1963, Decker and Sammek 1964) The position of the label of used ^{14}C-nicotine in this investigation, however, makes it impossible to detect demethylated products by autoradiographical means Experiments were performed in order to trace these compounds by their positive Koenig colour reaction However, with used chromatographic solvents the separation of the cofactor nicotinamide also giving positive Koenig reaction from demethylcotinine and nornicotine was not accurate enough to give quite reliable data

To get further information about the importance of demethylating pathways in liver metabolism of nicotine the appearance of ^{14}C-carbondioxide was measured The appearance of carbondioxide can be taken as a measure of the overall magnitude of demethylating pathways Of course the direct demethylation by trans methylation cannot be measured The methyl-group of nicotine is a precursor to respiratory carbondioxide and it is shown that 10—15 % of injected activity as nicotine-methyl-^{14}C is recovered as carbon dioxide in respiratory air in mice and rats during a 24 hours' period (McKennis *et al* 1962, Hansson and Schmiterlow 1962)

In incubations with nicotine there was a release of ^{14}C carbon dioxide Nevertheless no ^{14}C carbon dioxide could be observed in incubations shorter than 30 minutes This may indicate that the carbon dioxide is not derived directly from nicotine but from an intermediate metabolite

The absence of $^{14}CO_2$ after incubation with (±) cotinine-2-^{14}C is in correlation with findings by Morselli *et al* (1967) who could not detect any $^{14}CO_2$ in the expired air of rats given (±)-cotinine-2-^{14}C

Since numerous restrictions apply to the interpretation of the current data which were obtained with *(±) cotinine-2 ^{14}C, a study of the metabolism of the appropriately labelled*, naturally occurring L (—) form appears to be warranted

The similar pattern of nicotine and cotinine metabolites observed in in vitro and in vivo experiments suggests that the biotransformation of nicotine in the liver represents the major metabolic route in vivo

The present in vitro studies lead to the suggestion of the following sequence of reactions in the metabolism of nicotine and cotinine by the mouse liver (Fig 10)

This work was supported by grants to Professor Carl G Schmiterlow from the Swedish Tobacco Company the American Medical Association Education and Research Foundation, and Knut och Alice Wallenbergs Stiftelse (for general equipment for isotope research work)

The skilful technical assistance of Dr P Slanina, Mrs A Österblom and Mrs B Haeton is gratefully acknowledged

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Secretive Pressure Exerted by the Stimulated Nasal Salt Gland in Larus Argentatus (Herring Gull)

By

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Abstract

Håkansson, C H and B Malcus *Secretive pressure exerted by the stimulated nasal salt gland in Larus argentatus (herring gull)* Acta physiol scand 1970 78 249—254

Investigations of the dynamics of the nerve stimulated nasal salt gland of the *Larus argentatus* (Herring Gull) have shown that intraglandular duct anastomoses permit the gland to secrete through one duct if the other is occluded The force to extrude the secretion by maximal stimulation and uncatheterized ducts is lower than 40 mm H_2O The pressure force when both ducts were occluded was measured to 430 mm H_2O maximally

It was shown in a previous investigation (Håkansson and Malcus 1969) that electrical stimulation of the secretory nerve to the salt gland of the herring gull *Larus argentatus,* caused a rise in the intraglandular pressure It is necessary to consider the existence of the two ducts of the gland since some findings indicate intraglandular anastomoses In order to find out the dynamics within the gland this paper is concerned with direct measuring of the pressure from each duct as well as indirect measuring of the pressure exerted by the flow upon electrical nerve stimulation

Method

Larus argentatus was used in the experiments Totally 18 birds were used The operational technique the recording device and the experimental procedure have been fully described in a previous paper (Håkansson and Malcus 1969) Stimulation was 5 V, 30/sec 1 msec during 3 min if not otherwise stated

Experimental situations Fig 1 explains the experimental conditions The catheters connected to the flow meter tubing had a length of 7 mm and were made to fit the duct openings precisely

Results

Pressure calculated from flow In Fig 2 two flow curves of different recording situations and their derivatives (pressure curves) are shown The upper left flow curve is

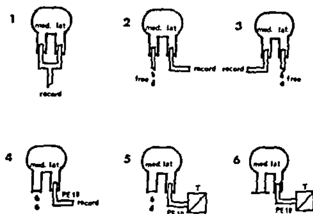


Fig 1 Experimental conditions
1 Flow recording (both ducts) Med duct 0 35 mm o d, 0 15 mm i d catheterization Lat duct 0 35 mm o d 0 15 mm i d catheterization
2 Flow recording (lateral duct) Med duct 0 35 mm o d, 0 15 mm i d catheter left open Lat duct 0 35 mm o d. 0 15 mm i.d catheterization
3 Flow recording (medial duct) Med.duct 0 35 mm o d, 0 15 mm i d catheterization Lat duct 0 35 mm o d, 0 15 mm i d. catheter left open
4 Flow recording (lateral duct) Med duct uncatheterized Lat duct 0 6 mm o d, 0 3 mm i d catheterization
5 Pressure recording (lateral duct) Med duct uncatheterized Lat duct 0 6 mm o d, 0 3 mm i d catheterization
6 Pressure recording (lateral duct) Med duct occluded Lat duct 0 6 mm o d, 0 3 mm i d catheterization
T = Pressure transducer PE 10 = Polyethylene catheter No 10

consistent with a secretion pressure of 150 mm H_2O The upper right flow curve corresponds to 37 mm H_2O maximally

Table I shows the collected results from Situations 1—4 in 4 birds The following conclusions can be drawn the secretion pressures in Numbers 1 2 and 3 are within the same range This is fully understood in view of the previously described findings that 50 per cent secretion is delivered from each duct (Håkansson and Malcus 1969) The pressure necessary to extrude the secretion in these cases lies between 185 and 100 mm H_2O and is larger than for no 4 (about 40 mm H_2O) The difference depends on the larger catheter diameter used in no 4 together with the free medial passage *The flow rate in all cases is mainly the same*

Pressure directly measured A pressure transducer (Sanborn 268B) connected to the lateral duct recorded maximum pressures exerted by the gland at nerve stimulation with the medial duct either free (Situation 5) or closed (Situation 6)

Experiments referring to Situation 5 gave the curves shown in Fig 3 the pressure rise is relatively slow up to a level of 300—350 mm H_2O which is reached after about 2 5 min stimulation Rhythmical pressure changes are superimposed on the curve They can be detected already after 1 5 min stimulation having a steadily increasing amplitude reaching its maximum after 3 min stimulation This amplitude is maintained until the end of stimulation. The oscillations have a slowly rising phase and a more abrupt decreasing phase Their frequency was found to be 5—5 5 c/min

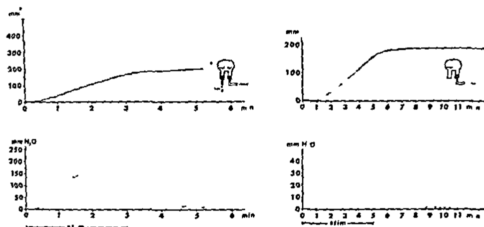


Fig 2 Left Flow rate recording referring to Situation 2 and its corresponding pressure change (stippled curve)
Right Flow rate recording referring to Situation 4 with corresponding pressure (stippled curve)

and the maximal amplitude 50 mm H_2O At the end of stimulation the fluctuations disappeared and the pressure rose to 420 mm H_2O followed by a slow poststimulatory declination Rhythmicity with the same frequency was found in two more birds We have no explanation for these rhythmic variations in the pressure

Another bird gave the type of pressure curve shown in Fig 4 This figure shows a first pressure maximum after 1 min at 132 mm H_2O The maximum pressure was reached after between 2 and 3 min of stimulation and was estimated to be 175 mm H_2O After the cessation of stimulus the pressure level slowly decreased to 100 mm H_2O

TABLE I

Recording Situation no	Bird no	Maximal Flow Rate mm³/sec	Secretion Pressure mm H_2O
1	I	2 00	185
	II	1 85	170
2	I	0 81	150
	II	0 81	150
3	I	0 80	148
	II	0 82	103
4	III	0 82	40 0

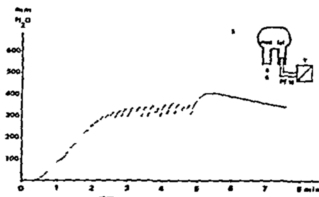


Fig 3 Pressure recording referring to Situation 5 Regular rhythmical fluctuations are superimposed on the pressure curve

Further at the beginning of stimulation this curve like many others showed an initial fall in pressure This dip is clearly seen in the inset in Fig 4 and amounts to 3 mm H_2O during the first 3 sec. In other recordings this dip could have a duration of 15 sec but its magnitude seldom surpassed 4 mm H_2O In some curves the dip was not observed at all

Situation no 6 is illustrated by Fig 5 With the medial duct closed the pressure rises after 15 sec latency to 430 mm H_2O This maximum pressure is attained after 1 5 min of stimulation Pressure fluctuations were not clearly seen The level rather sinks slowly to 385 mm H_2O at the end of stimulation (5 min) Thereafter the pressure decrease continued to a constant level of 120 mm H_2O

The rate of pressure rise in Situation no 6 was measured to 10 mm H_2O sec which is the highest value obtained in any of these experiments

Since the results above might indicate intraglandular anastomoses contrast medium (barium solution was injected in retrograde direction through the lateral duct opening It soon flowed out through the medial duct opening X ray picture (Fig 6 of the gland is a proof of anastomoses between the ducts

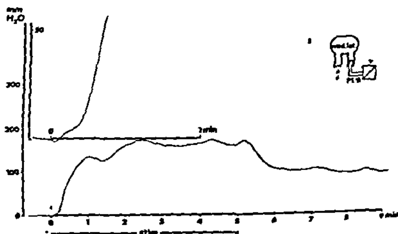


Fig 4 Recording of pressure Situation 5 Note the initial dip amplified in the inset.

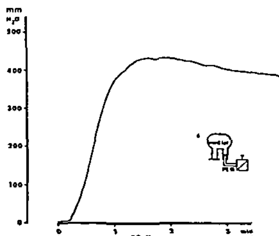


Fig 5 Maximum pressure recording Situation 6

Discussion

The secretion from the salt gland flows out through two ducts These ducts anastomose within the gland permitting its drainage even if one duct is closed This is concluded from the determinations of the maximum pressure from the lateral duct with the medial duct either free or occluded (Fig 3—5) The final proof is the radiograph of the ducts (Fig 6)

The secretion pressure at open outflow is determined by the size of the catheters The gland however is capable of secreting the same volume/time unit in response to a given stimulation even if it has to overcome a greater resistance The secretion pressure was found to be about 40 mm H_2O at 0 3 mm i d catheterization of the



Fig 6 Radiograph of the salt gland with ducts and intraglandular anastomoses

gland However this is far from its maximum performance With the lateral duct occluded and the medial left open an intraglandular pressure of 200—300 mm H_2O was recorded It rose to > 400 mm H_2O if the medial duct was closed as well

The pressure of the salt gland is to be compared with those of other glands Yoshimura *et al* (1962) found a secretory pressure of about 150 mm Hg (2010 mm H_2O) in the dog s submaxillary gland at stimulation of the *chorda tympani* In the human parotid gland the same authors found a secretion pressure of 12—13 mm Hg (~ 175 mm H_2O) at chemical stimulation of the tongue Maximum pressure determinations in the human parotid gland have shown up to 600 mm H_2O (Håkansson and Lipecki to be published)

The rise in pressure was often preceded by an initial deflection of the base line indicating a negative pressure This observation means a widening of the glandular lumen. This might involve vasoconstriction or activity of myogenic elements like basket cells Such cells however have not hitherto been shown in the salt gland as they have in many salivary glands

Fange *et al* (1958) showed that stimulation of the nerve containing the secretory fibres to the salt gland indicated the presence of sympathetic fibres since the feathers on the top of the head responded as on sympathetic nerve stimulation (Langley 1903) Sympathetic fibres have not been shown to enter the gland but Cords (1904) has shown that *gangl ethmoidale* is connected with sympathetic fibres

The expenses for the investigations have been defrayed by grants from B Kamprads Fund for Medical Research and by grants from the University of Gothenburg

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Acta physiol. scand. 1970 78 255—268

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The Distribution of Sodium, Potassium and Chloride in the Smooth Muscle of the Rat Portal Vein

By

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Abstract

HALJAMÄE, H, B JOHANSSON, O JONSSON and H ROCKERT *The distribution of sodium, potassium and chloride in the smooth muscle of the rat portal vein* Acta physiol scand 1970 *78* 255—268

The contents of Na and K in isolated preparations of the rat portal vein were determined by micro flame photometry and the distribution volumes of ^{24}Na ^{42}K and ^{36}Cl in this vascular muscle were measured after incubation periods of variable duration. The results indicate the following total ion concentrations in the vessel wall Na 94 5, K 51 2 and Cl 100 meq/kg wet weight. Evidence was obtained for the existence of "bound fractions of the three ions and a series of experiments was designed to determine the size of these extra compartments Data from these experiments and measurements of total water and of extracellular space by sucrose ^{14}C allowed calculation of intracellular ion concentrations. The following values in meq/l intracellular fluid were obtained $[Na^+]_i$ 44 8 $[K^+]_i$ 138 and $[Cl^-]_i$ 86 5 The equilibrium potentials for the three ions would then be +30 —84 and —12 mV, respectively Functional implications of this ionic distribution are discussed

Measurements of total K contents and of extracellular space in portal vein preparations incubated in hyperosmotic solution (Krebs +150 mmoles sucrose/l) indicated a clear-cut increase of $[K^+]_i$ due to osmotic dehydration of the cells A net loss of K seemed to occur with long periods of incubation in hyperosmotic solution

The ionic composition of the vascular walls has been the subject of numerous investigations, often initiated by the authors interest in the possible importance of electrolyte disturbances in the development of hypertensive vascular disease (*e g* Tobian and Binion 1954 Heading Rondell and Bohr 1960, Friedman and Friedman 1965 Jones, Feigl and Peterson 1964 Villamil *et al* 1968 a) It is evident that such studies are of great importance also for the understanding of the basic physiology of vascular smooth muscle For instance the electrical membrane characteristics of the muscle cells must be considered in the light of information about the distribution of ions between the intra and extracellular spaces A problem is that studies of the ionic composition of vascular walls have been performed mainly on large and medium sized arteries where the electrical characteristics of the smooth muscle are still quite obscure whereas information about ionic distribution seems to be lacking

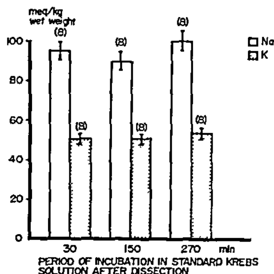


Fig 1

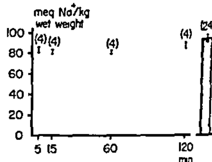


Fig 2

Fig 1 Total contents of Na and K in rat portal vein determined by flame photometry after different periods of incubation in normal Krebs solution The ion concentrations are expressed as meq/kg wet weight Vertical bars represent ± S E M

Fig 2 Uptake of ^{24}Na in the isolated rat portal vein compared to the total contents of Na (shaded column) determined by flame photometry The distribution volumes of the tracer ion after the different periods of incubation have been converted to meq/kg wet weight Vertical bars represent ± S E M

between 150 and 270 min is uncertain (0 1>p>0 05) It thus appears that the muscle is in a relatively steady state with regard to its ion contents over the entire period studied The three sets of data have therefore been analysed together resulting in Na and K contents of 94 5 ± 2 5 (S E) and 51 2 ± 1 1 meq/kg wet weight respectively These figures will be given as control values for some of the experiments reported below

2 *Uptake of ^{24}Na ^{42}K and ^{36}Cl*

Fig 2 3 and 4 illustrate the results of experiments in which the uptake of ^{24}Na, ^{42}K and ^{36}Cl in isolated portal vein preparations was studied The distribution volumes of the isotopes after different periods of incubation have been converted to ionic concentrations in meq/kg wet weight The uptake data for ^{24}Na and ^{42}K can then be compared with the results of the flame photometry which are represented by the shaded columns in Fig 2 and 3 It can be seen that after 5 min ^{24}Na is already distributed in a space which corresponds to about 90 per cent of the total Na content A subsequent slow component is likely to be present in the uptake of ^{24}Na but it cannot be clearly distinguished in these data With ^{42}K the uptake curve shows first a small rapid component which can probably be attributed in great part to diffusion into the extracellular space and then a slow phase with a $t_{1/2}$ of about 34 min After 6 hrs of incubation when the uptake curve seems to approach a

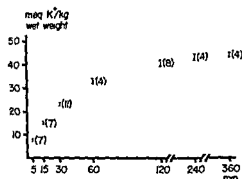


Fig 3

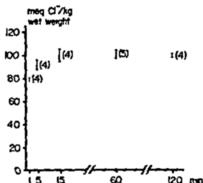


Fig 4

Fig 3 Uptake of ^{42}K in the isolated rat portal vein compared to the total contents of K (shaded column) determined by flame photometry The distribution volumes of the tracer ion after the different periods of incubation have been converted to meq/kg wet weight Vertical bars represent ± S E M

Fig 4 Uptake of ^{36}Cl in the isolated rat portal vein The distribution volumes of the tracer ion after the different periods of incubation have been converted to meq/kg wet weight Vertical bars represent ± S E M

plateau, the ^{42}K space is still clearly below the level of total K as measured by flame photometry This difference could be due to the presence of a very slowly exchangeable fraction of K or perhaps to a failure of the muscle preparations to maintain a normal K uptake over the longer incubation periods of the tracer experiments Despite this difference it can be said, in general that the results from flame photometry and the "ultimate" distribution volumes of the tracer ions agree satisfactorily, so that the results of the two types of experiments support each other

Total Cl content in the portal vein has not been measured in the present study, but the plateau of the uptake curve in Fig 4 may suggest that ^{36}Cl has equilibrated with the total Cl space during the long incubation periods The level of this plateau will be taken to represent total Cl even though a reservation has to be made for the possible existence of an additional slowly exchangeable fraction The uptake curve indicates a Cl content of 100 meq/kg wet weight

3 *Total water and extracellular space*

Measurements of total water and extracellular volume (sucrose ^{14}C space) were performed to allow calculations regarding the distribution of the ions in the muscle The total water content measured by drying to constant weight was 79 0 ml/100 g wet weight for portal veins incubated in normal Krebs solution (see Arvill *et al* 1969) Extracellular fluid determined as sucrose ^{14}C space after 15 min of incubation was 49 3 ml/100 g wet weight in the present study Lower mean values 45 4 and 42 8 ml/100 g respectively have been reported previously (Arvill *et al* 1969, Jonsson 1969 b) but these were obtained in experiments where the muscles were briefly washed in non radioactive solution after incubation This procedure can be

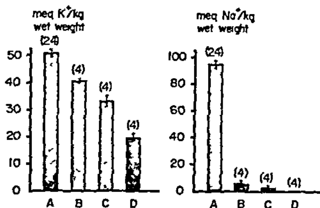


Fig 5 Total contents of K and Na in the rat portal vein determined by flame photometry after incubation in normal Krebs solution (A) and in isotonic K Na free sucrose solution containing 2 5 mmoles $CaCl_2$/l for 5 30 and 60 min, respectively, (B, C D Vertical bars represent ± S E M

justified if one wants to exclude the fluid layer at the surface of the tissue from the extracellular space However, since this fluid layer is included in the "total water" and since its ion contents influence the data from flame photometry, it should also be included in the extracellular volume when this is to be used in calculations of ionic distribution Therefore sucrose ^{14}C space was determined in the present study without any "washing" between incubation and blotting This washing procedure was also omitted in the experiments on uptake of radioactive ions

4 Calculation of intracellular ion concentrations

If the results presented under section 1—3 above are used for calculating the amounts of intracellular ions according to a conventional two compartment tissue model the following mean values are obtained $[Na^+]_i = 26.9$ meq/kg wet weight or 90 meq/l intracellular fluid, $[K]_i = 48.3$ meq /kg wet weight or 163 meq/l intracellular fluid, $[Cl]_i = 33.9$ meq/kg wet weight or 114 meq/l intracellular fluid These intracellular concentrations if represented entirely by free ions are not compatible with an osmotic balance over the cell membranes

It seems necessary therefore to consider the possible existence of bound electrolytes in the tissue which implies in principle that a third compartment must be included in the tissue model

5 'Bound' ions

One possible explanation for the high Na contents of the portal vein would be the presence of a 'sequestrated amount of this electrolyte in paracellular or intracellular structures However the uptake curve for ^{24}Na which rapidly approached values corresponding to the total Na content determined by flame photometry (Fig 5) speaks against the existence of any larger fraction of slowly exchangeable Na Further evidence against any sizeable component of firmly bound Na was obtained in the following experiments Sodium and potassium were measured by flame photometry in 12 tissue samples which had been incubated for variable periods of time in an

		Freezing + thawing		Cyanide + iodoacetate	
NORMAL KREBS	Total H_2O	79.7±1.6	(6)	82.2±2.8	(6)
	Sucrose-^{14}C space	76.6±0.8	(5)	84.7±1.7	(6)
	^{24}Na space	93.1±1.0	(6)	97.5±1.6	(6)
	^{42}K space	93.1±1.4	(5)	94.7±0.4	(5)
	^{36}Cl space	86.8±1.4	(6)		
"HIGH K^+ KREBS"	^{42}K space	91.2±0.9	(6)		

Fig 6 Total water contents and distribution volumes for sucrose ^{14}C ^{24}Na ^{42}K and ^{36}Cl in rat portal veins where the cell membranes were damaged by freezing and thawing or by poisoning with sodium cyanide and mono-iodo-acetic acid The incubation was performed either in normal Krebs solution or in a Krebs solution where NaCl had been replaced by KCl All values are given in *ml/100* g wet weight

isotonic solution of sucrose with 2 5 mmoles $CaCl_2$/l After the usual pre incubation for 30 min in normal Krebs following the dissection, the preparations were divided in three groups which were kept in the K Na free solution for 5, 30 and 60 min respectively As shown in Fig 5 there was an abrupt and pronounced fall in Na to about 6 per cent of the control value in the first 5 min and a further decline there after In 60 min the Na contents had fallen below the measurable level Potassium showed a less dramatic decrease

If a substantial fraction of the total Na should be intracellular, either as free ions or loosely bound , the rapid fall in Na requires that its passage through the cell membranes should be greatly facilitated by some mechanism not available to the free intracellular K Another explanation would be that the excess Na resides at extracellular sites from which it is readily released when $[Na]_o$ is lowered a possibility which may appear more attractive

Another series of experiments was designed to obtain further information about the ionic compartments which seemed to exist besides the conventional intra and extracellular fluids Experiments were performed on muscles which were either frozen and thawed or subjected to poisoning with cyanide and iodoacetate (for details see Methods) The purpose of these procedures was to destroy the barrier function of the cell membrane so that the entire fluid phase of the tissue would become homogenous and equilibrated with the external medium Such preparations were studied with regard to total water sucrose ^{14}C space ^{24}Na space ^{42}K space and ^{36}Cl space *The results are summarized in Fig 6 A small increase in total* water in comparison with normal muscles (79 0 ml/100 g) is observed with both types of preparations but the change is statistically significant only for the cyanide iodoacetate group The sucrose space is practically equivalent to total water showing that sucrose which

the value for muscles in normal solution 49 3 ml/100 g reported in section 3 above An approximate calculation of intracellular K^+ can then be made for the 15 min period of hyperosmolarity if the proportion of "bound" K under these conditions is assumed to be the same as in normal muscles A value for $[K]_i$ of 223 meq/l intracellular fluid is obtained This implies an increase of $[K]_i$ by about 62 per cent compared to the control (section 6 above) and since theoretically, an increase by 53 per cent should have occurred with the actual degree of hyperosmolarity the result seems quite compatible with an osmotic behaviour of the vascular smooth muscle cells

After 60 min in hypertonic medium there appears to be a marked decrease in the K content (Fig 8D) suggesting a net loss of K from the tissue Extracellular space and total water have not been determined for this situation and it is not possible therefore to calculate the compartmental potassium distribution However a loss of intracellular K as a result of the increased transmembrane concentration gradient after the long lasting exposure to hyperosmolarity seems quite likely

The Na content of the muscles after different periods of hyperosmolarity was also measured by micro flame photometry However, the results were erratic and showed no consistent trends which may be due to variations in extracellular space or methodological difficulties with the blotting procedure when the muscles were taken from the sucrose rich solution Such factors would of course have a marked influence on the measurements of substances with predominantly extracellular localization The data that we have obtained so far do not seem to allow any conclusions as to the shifts in the Na distribution of the portal vein on exposure to hypertonic solutions

Discussion

The ionic composition of arterial smooth muscle has been studied extensively but the functional implication of the distribution of the ions across the cell membrane is not clear since the electrophysiological characteristics of this kind of muscle are largely unknown Preparations of vascular smooth muscle from splanchnic veins have been used successfully in recent years for electrophysiological studies with both extracellular and intracellular recording techniques (e g Funaki and Bohr 1964 Cuthbert and Sutter 1965 Axelsson et al 1967 Nakajima and Horn 1967) Studies on the isolated rat portal vein have demonstrated the consistent relationship between electrical and mechanical activity in this propagating vascular muscle (Funaki and Bohr 1964 Axelsson et al 1967 Johansson et al 1967 Johansson and Jonsson 1968 a b) It seems therefore that knowledge of the ionic distribution in this kind of preparation would reveal more clearly the roles of the different ions in the function of vascular smooth muscle

From the methodological point of view the portal vein preparation presents certain advantages with regard to the exchange of tracer substances due to the short diffusion distances in the thin vessel wall On the other hand the same factor

implies difficulties with regard to the standardization of the dissection and blotting procedures which may contribute to the scattering of the data obtained

The present results are relevant only for the ionic composition of the muscle under the actual *in vitro* condition and may not describe exactly the normal situation *in vivo* However, the functional studies of electrical and mechanical activity referred to above have also been carried out *in vitro*, and these have shown that the muscle maintains spontaneous activity and responsiveness for several hours under circumstances comparable to those of the present experiments

The present study indicates that the total contents of Na and Cl in meq/kg are high in the venous smooth muscle, whereas K is low in comparison with tissues such as skeletal muscle It appears as if the portal vein in this respect deviates from skeletal muscle even more than does visceral smooth muscle (for ref see *e g* Burnstock, Holman and Prosser 1963, Goodford 1968) The ionic composition of the venous wall resembles that reported for arterial preparations (*e g* Hagemeijer, Rorive and Schoffeniels 1965, Villamil *et al* 1968 a, b)

Wahlstrom recently studied the ionic composition of the isolated rat portal vein using atomic absorption techniques (Wahlström, personal communication) He found total Na and K contents of about 87 and 52 meq/kg wet weight respectively for muscles incubated in normal Krebs solution These data agree satisfactorily with those reported in section *I* above, although the sodium contents were somewhat higher in the present study Wahlstrom also studied the uptake of radioactive potassium and found a difference between the ^{42}K space at long periods of incubation and the total K contents similar to that shown in Fig 3 above

In calculating the distribution of the ions in the different compartments of the tissue from the present results we are faced with the same problems as other investigators working with smooth muscle namely the poor definition of the extracellular space and the difficulties in quantitating the bound or non exchangeable ion fractions Different values for extracellular space have been obtained depending on the tracer substance used (*e g* Burnstock *et al* 1963 Barr and Malvin 1965) but sucrose-^{14}C which we have used in this and previous studies (Arvill *et al* 1969 Jonsson 1969 b) has become widely accepted because of its ability to penetrate rapidly into the extracellular space and its apparently effective exclusion from the cell interior It also seems as if binding of sucrose to the tissue does not occur to any significant extent (Fig 6) It seems clear that the ionic distribution in smooth muscle cannot be fully described on the basis of a two-compartment tissue model A third compartment represented by 'bound or sequestrated ions seems to exist besides the extra and intracellular fluid compartments The experimental approach used for determining the extent of this binding in vascular preparations has consisted of destroying the normal membrane barriers and measurement of the amounts of ions in the tissue exceeding those dissolved in the fluid phase (*e g* Heading Rondell and Bohr 1960 Friedman and Friedman 1967 Villamil *et al* 1968 a) This approach has also been applied in the present study although the amounts of excess ions have been evaluated on the basis of long term uptake of radioactive Na, K

and Cl It is not certain whether the data *from the damaged muscles can be transferred* directly to the normal tissue, since the relative size of the bound ion fractions might be influenced for instance by the cell metabolism The values given for the magnitude of the bound compartments (Fig 7) should be considered with these reservations in mind Some authors have assumed in their calculations that the bound cations are entirely extracellular (Heading Rondell and Bohr 1960, Villamil *et al* 1968 a) whereas others have suggested both intra and extracellular localizations (*e g* Friedman and Friedman 1967) In the present study a ratio of free to total ions has been obtained from the frozen and thawed muscles and this ratio has been applied to the total ion content of normal muscles in order to calculate the size of their bound compartments The bound fractions determined with the present method are characterized by a relatively easy exchange with the free ions The difference between the plateau of the ^{42}K uptake and total K determined by flame photometry might indicate the existence of another, more firmly bound K fraction If this were so the size of the bound K compartment in Fig 7 would increase at the expense of the free intracellular K^+ As the total Cl contents of the portal vein have not been measured directly but calculated on the basis of the ^{36}Cl uptake we cannot exclude the possibility of non exchangeable Cl fractions Such a compartment has been suggested for arterial smooth muscle (Villamil *et al* 1968 b)

The ionic distribution of portal vein illustrated in Fig 7 results in equilibrium potentials for Na and K of +30 and −84 mV respectively If the membrane potential which seems to average about −50 mV (Funaki and Bohr 1964 Nakajima and Horn 1967) is determined essentially by the transmembrane gradients and permeabilities of these ions according to the constant field equation (Goldman 1943) the ratio of P_{Na}/P_K would be about 0 12 The calculated equilibrium potential for Cl is considerably lower than the above value for the membrane potential which may indicate that this ion is not passively distributed across the membrane although some deviation in this direction is to be expected as a result of the spontaneous muscle activity With regard to the distribution of Cl in intestinal smooth muscle there is some difference of opinion as to whether this conforms to a passive Donnan equilibrium or whether there is an active transport of *Cl* (Buck and Goodford 1966 Casteels and Kuriyama 1966)

Previous work (Arvill *et al* 1969) has shown that the smooth muscle cells behave as perfect osmometers on increases in extracellular osmolarity and it may therefore be of interest to consider the concept of osmotic balance over the cell membranes in the light of the present results *The osmolarity of the normal* Krebs solution is 284 mOsm/l The sum of the concentrations of Na K and Cl in the intracellular fluid (Fig 7) amounts to 269 meq/l which should correspond to a somewhat lower numerical value for osmolarity in mOsm/l However the sum of $[Na]_i$ and $[K]_i$ exceed $[Cl]_i$ by 96 meq/l and this extra amount of negative charge is thus required for intracellular electro-neutrality An osmotic balance between extra and intracellular fluid could be obtained if the extra negative charge were represented by polyvalent particles or if the osmotic coefficient of the intracellular solutes was low

Otherwise the intracellular osmolarity would exceed that of the extracellular fluid and the corresponding hydrostatic pressure would have to be opposed by the tension of the cell wall

Since many problems remain unsolved with regard to the ionic distribution in the portal vein in normal Krebs solution, it is evident that the calculations based on the results obtained from muscles in hyperosmotic medium must be interpreted with caution It seems clear, however, that there is a primary increase of $[K^+]_i$ to an extent expected for osmotic cells The resulting increase of the ratio $[K^+]_i/[K^+]_o$ could evidently contribute to the hyperpolarization caused by hyperosmolarity (Johansson and Jonsson 1968 a) Evidence was obtained however in this earlier study that this factor cannot solely account for the change in electrical activity If the decrease in total K observed after a long exposure to hyperosmolarity (Fig 8 D) represents a net loss of intracellular K^+ this could help to explain the excitation of muscle activity seen on return to normal Krebs solution after long periods in hyperosmotic sucrose Krebs (Arvill *et al* 1969)

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Effects of Noradrenaline upon Cerebrocortical Activity in Asphyxiated Newborn Rabbits

By

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Abstract

BOËTHIUS, J, T BRUNDIN and N-Å PERSSON *Effects of noradrenaline upon cerebrocortical activity in asphyxiated newborn rabbits* Acta physiol scand 1970 *78* 269—273

The asphyxia resistance of the direct cortical response (DCR) and the electrocorticogram (ECG) was determined in noradrenaline treated and untreated newborn rabbits The resistance of the untreated animals was similar to what has previously been found for the adult the DCR being blocked within 3 1/2 min and the ECG within 2 min of asphyxia After the administration of noradrenaline the DCR became more resistant to asphyxia and could be evoked for 8—17 min The ECG was not sustained by noradrenaline and was blocked within 2 min of asphyxia as it was in the untreated animals

An increased ability of the DCR to withstand asphyxia has been described for the fetus

The direct cerebrocortical response (DCR) is a characteristic sequence of potentials which can be recorded from the surface of the cerebral cortex in response to an electrical stimulation applied to the cortex at a nearby point (Adrian 1936 and others) It is well known from studies in adult animals that the electrical activities of the cerebral cortex are readily blocked by any deterioration of the oxygen supply to the brain This effect holds true for the electrocorticogram (ECG) and for the DCR as well (*cf* Chang 1951) On the other hand, corresponding to the well known ability of the fetal central nervous system to withstand asphyxia the DCR has proved to be remarkably resistant to asphyxia when recorded from the fetal cerebral cortex Thus in deeply asphyxiated fetal lambs the DCR could be evoked for considerable periods of time, whereas the ECG was extinguished after a few minutes of asphyxia (Meyerson 1964)

The greater ability of the immature central nervous system to withstand the effects of asphyxia has been ascribed to the relatively greater role of anaerobic glycolysis in

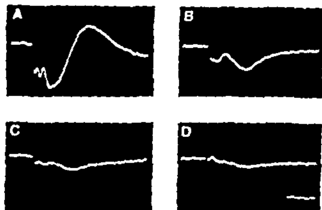


Fig 1 Effect of asphyxia on the DCR of a newborn rabbit A before asphyxia B 2 min C 3 min and D 3 1/2 min after clamping the tracheal cannula Time bar 30 msec

the brain of fetal and newborn mammals (Chesler and Himwich 1944) but otherwise the mechanisms underlying this resistance are unkonwn It has been found however that in both fetal and newborn mammals the condition of asphyxia consistently leads to a release of large amounts of noradrenaline from the preaortal paraganglia (Brundin 1966) and from the adrenaline medulla (Comline and Silver 1958) The present study was undertaken in order to investigate the effects of noradrenaline on the asphyxia susceptibility of the DCR and the ECG of newborn rabbits

Methods

The experiments were performed on 16 newborn rabbits (age less than 3 days) The animals were tracheotomized under ether anesthesia immobilized with Flaxedil 0 4 mg/h b w s c) and ventilated artificially A unilateral craniotomy was performed and the cortical surface of one of the cerebral hemispheres was exposed and covered with warmed mineral oil

The DCR was induced by stimulating the cortical surface with supramaximal square waves (0 15 msec The stimulus was repeated every 30th second and delivered through a bipolar electrode The DCR was recorded with a monopolar Ag AgCl electrode placed 3—5 mm from the stimulatory electrode and with a reference electrode on the skull The signal was then fed into a DC-coupled preamplifier displayed on a cathode ray oscillograph and photographed The ECG was recorded with Ag AgCl electrodes or with saline bridges linked to matched calomel half cells and registered on an EEG machine (Grass Mod III time constant 125 msec)

Asphyxia was induced by stopping the artificial ventilation and clamping the tracheal cannula. Immediately before the onset of asphyxia each experimental animal received a large dose of noradrenaline 80—200 ng/g b w s c The controls got corresponding volumes of saline

Results

The DCR obtained from the control animals consisted of two negative components one fast primary and one slow secondary potential (Fig 1 A and 2 A) The total duration of this response was 100—200 msec Similar characteristics of the DCR have been reported previously (Do Carmo 1960)

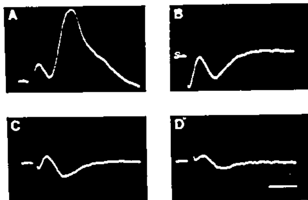


Fig 2 Effect of asphyxia on the DCR of a newborn rabbit pretreated with noradrenaline (200 ng/g b w) A before asphyxia B 10 min C 15 min and D 17 min after clamping the tracheal cannula. Polarity of the stimulation artifact changed between A and B Time bar 30 msec

When the animals were asphyxiated, the DCR disappeared within 3 1/2 min (Fig 2 A—D) This time was also the measure of the asphyxia resistance of the primary component which was always found to be the most resistant part of the DCR The secondary component usually disappeared after 2 min of asphyxia These time values for the asphyxia susceptibility of the DCR do not differ from those described for the adult rabbit (*cf* Chang 1951)

When noradrenaline was administered there was a considerable increase of the time during which the DCR could be evoked This increased resistance to asphyxia could be demonstrated both for the primary component which was recorded for 8—17 min, and for the secondary component which remained for 5—8 min after the onset of asphyxia Apart from this increase of the resistance to asphyxia, the general features of the DCR did not seem to be changed by the administration of noradrenaline, and neither before nor during asphyxia could any difference be detected between the two groups in regard to time course or configuration of the response

The ECG curves obtained from the control animals were dominated by slow rhythms and showed the same general characteristics as have been described for newborn rabbits by others (*cf* Garma 1966) Within 2 min of asphyxia the ECG disappeared completely Noradrenaline treatment did not increase the asphyxia tolerance of the ECG The ECG curves from the noradrenaline treated animals did not differ qualitatively from those of the controls In some experiments the ventilation was restarted 10 min after the ECG had initially disappeared Spontaneous electrical activity then revived in the form of bursts of waves of high amplitude and long duration These bursts increased in frequency and decreased in amplitude and eventually a continuous ECG was established resembling that obtained before the profund asphyxia. This restoration could be observed in both noradrenaline treated and control animals A detailed study of the effect of asphyxia on the ECG will be published separately

Discussion

In the present study the administration of noradrenaline caused the DCR of new born rabbits to be markedly more resistant to asphyxia The ECG, however, was not sustained by this treatment Thus, regarding the asphyxia resistance of the DCR and ECG, untreated newborn rabbits reacted in a manner similar to that of the adult Noradrenaline administration, however, instigated an asphyxia tolerance of the DCR similar to that described for the fetus

This effect of noradrenaline seems interesting since it has been shown that in direct response to asphyxia fetal and newborn mammals release large amounts of this hormone from the preaortal paraganglia (Brundin 1966) and from the adrenal medulla (Comline and Silver 1958, 1966) In the light of this evidence it is tempting to speculate that the increased asphyxia resistance of the fetal DCR is in some way associated with the effects of noradrenaline released from the preaortal paraganglia (which degenerate after birth) and from the adrenal medulla Since such an asphyxia induced noradrenaline release is known to occur in the newborn animal as well as in the fetus, an asphyxia resistant DCR would be expected also in the new born In the present experiments the physiologically released noradrenaline did not seem to be sufficient for this effect although it was easily demonstrated when an additional dose of noradrenaline was administered However, for a proper inter pretation of these results the circulatory changes at birth must be taken into con sideration, since the establishment of an effective pulmonary circulation prevents blood from the noradrenaline secreting organs to reach the brain directly and with out admixture The venous blood from both the preaortal paraganglia and the adrenal medulla drain into the inferior vena cava In the fetal circulation the blood of the inferior vena cava is mainly passed through the foramen ovale to the left heart and immediately ejected into the systemic arteries On the other hand after birth the systemic venous blood from both cavae is mixed in the right heart and passes through the pulmonary vessels before reaching the left heart and the arteries During this time consuming passage the blood concentration of noradrenaline would be expected to decrease through the effects of dilution and metabolic inactivation. The resulting lower arterial concentration of noradrenaline could possibly explain the failure to demonstrate any asphyxia resistant DCR in the newborn animals. Such an explanation seems likely particularly in view of the fact that an additional and very large dose of noradrenaline was able to restore the asphyxia resistance of the DCR

The mechanisms are obscure whereby noradrenaline effects this increase in asphyxia resistance of the DCR One possibility is a direct action of noradrenaline on the central nervous system Such a hypothetical direct action might be a mechanism governing the ratio of aerobic *versus* anaerobic metabolism Arguing against the possibility of such a process is the presence of a blood brain barrier for noradrenaline which according to isotope studies seems to be fully developed at birth in the rabbit (Brundin Lewander and Öhnell to be published) On the other

hand, in the present experiments the capacity of this barrier might have been exceeded by the high concentration of noradrenaline resulting in a transfer of noradrenaline to the central neurons. Further, asphyxia *per se* might have broken down the blood brain barrier allowing passage of noradrenaline into the brain tissue.

Another possibility is that the effect of noradrenaline on the asphyxia resistance of the DCR is indirect and due to the general metabolic and circulatory effects of this hormone. Thus it is known that in the newborn rat the survival time in anoxia can be prolonged by inducing hyperglycemia (Stafford and Weatherall 1960). Due to the well known glycemic effect of noradrenaline the blood glucose concentration would be elevated and hence, an increased survival time could be expected. Further, the vasoconstrictor effects of noradrenaline are relatively weak in the brain vessels (Sensenbach, Madison and Ochs 1953, and others) which together with the expected blood pressure rise would favour an enhanced blood distribution to the brain which would facilitate its oxygen supply at least during the first few minutes of asphyxia.

Obviously, more information is required to clarify the mechanisms underlying the noradrenaline induced asphyxia resistance of the DCR and work is in progress on the functional capacity of the blood brain barrier for noradrenaline in newborn mammals subjected to asphyxia.

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Acta physiol. scand. 1970 78 274—279

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Disappearance of Serum Insulin in Obese-Hyperglycemic Mice

By

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Received 30 April 1969

Abstract

COORE, H G and S WESTMAN *Disappearance of serum insulin in obese hyperglycemic mice* Acta physiol scand 1970 78 274—279

The disappearance from serum [illegible]
and lean mice Groups of mice [illegible]
or a tracer dose ^{125}I labelled [illegible]
body bound radioactive insulin [illegible]
was noted between the obese a[illegible]
more physiological conditions with a tracer dose, it was found that the rate of insulin disappearance from serum was greater in the obese animals These findings are discussed against the background of previous observations of increased resistance to insulin hypoglycemia in obese mice and an increased insulin degrading activity of adipose tissue homogenates from such mice

Animals which develop a spontaneous diabetes syndrome have been intensively studied in recent years in the hope that information relevant to human diabetes mellitus may thereby be obtained Interest has been focussed on the obese hyperglycemic syndrome in mice, and these animals have been widely used as experimental models A unit recessive autosomal gene with complete penetrance is regarded as responsible for the syndrome (Ingalls, Dickie and Snell 1950) A prominent feature of the obese mice is their marked insulin tolerance (Mayer *et al* 1953, Stauffacher *et al* 1967 Westman 1968a) There is as yet no generally accepted theory of the cause of this insulin tolerance In view of earlier observations that homogenates of adipose tissue from obese mice were eight times more effective in degrading insulin in vitro than similar tissue from lean littermates (Westman 1968b) it seemed important to test the possibility that a higher disappearance rate in vivo contributed to the insulin tolerance of the intact animal

Material and Methods

Animals Altogether 56 male mice 5 months old, were used in this study The animals belonged to the strain with the recessively inherited obese hyperglycemic syndrome, (gene symbol ob/ob) originally established at the Roscoe B Jackson Memorial Laboratory, Bar Harbor

Maine, U S A, and bred at the Histological Department University of Uppsala Sweden The mice were allowed free access to commercial pelleted food (AB Ewos, Sodertalje Sweden) and tap water (Westman 1968a) They were divided into the following groups and all investigations were performed in the morning

A Seventeen obese and 13 lean mice were used for the determination of the level of immunoreactive serum insulin

B Nine obese and 9 lean mice were injected in the tail vein with 600 U crystalline insulin/kg b w to study the rate of insulin disappearance from serum after a heavy insulin load Blood samples were taken within 1 min after the injection (zero time value) and after 2, 4, 6 10 and 30 min or after 6 15 30 45 and 60 min

C Four obese and 4 lean mice were similarly injected i v with a tracer dose of ^{125}I labelled insulin By administration of 80 μl solution containing 8 mμg insulin the insulin disappearance *was followed under more physiological conditions Blood samples were taken within 1 min after* the injection (zero time value) and after 4, 8, 16 and 32 min

Blood samples were obtained by puncture of the orbital vein plexus with a thin walled Pasteur pipette The blood was then collected in dry test tubes and allowed to coagulate, after centrifugation the serum was subsequently removed.

Insulin and antibody preparations Crystalline zinc insulin, 80 U/ml (Insulin Novo®, Novo, Copenhagen Denmark) was used for the load and the insulin immunoassay kit supplied by the Radiochemical Centre Amersham Bucks England was used for the determinations of the serum insulin concentrations ^{125}I labelled bovine insulin (approx spec act 50 mC/mg) (The Radiochemical Centre Amersham) was given as tracer dose Guinea pig anti bovine insulin serum prepared essentially as described by Robinson and Wright (1961) and precipitating rabbit anti guinea pig serum (Wellcome Brand Diagnostic Reagent Beckenham England) were utilized in part C of the study

the radioimmunoassay
n as a standard (ex
were carried out on
I count rates and the
error for means of duplicate insulin determinations was ±12 %. When only a tracer dose of ^{125}I labelled insulin was given the amount of immunoreactive labelled serum insulin was determined on duplicate samples of 5 μl serum by adding excess of guinea pig anti insulin serum to bind total exogenous and endogenous insulin The anti insulin serum was shown to bind the expected amount of labelled insulin in 5 μl serum without diminution by addition of 8 mμg of insulin The highest amount of endogenous mouse insulin in 5 μl serum was 6 mμg Appropriate amounts of precipitating anti guinea pig serum were then added and the precipitated complex filtered and counted as in the immunoassay

Glucose determinations Serum glucose was determined on samples of 5 μl by a glucose oxidase method (Hjelm and deVerdier 1963)

Results

The decline in serum insulin following injection of 600 U insulin/kg b w to the obese and lean mice is shown in Fig 1 For each animal the serum insulin level of the sample obtained at zero time was considered as 100 per cent and the subsequent samples expressed as fractions thereof The concentration of serum insulin at zero time was approximately the same in the obese and the lean animals (see Table I) Despite the high insulin levels none of the animals, which were well nourished displayed convulsions or was shocked during the experimental period In fact both groups of animals showed elevated zero time serum glucose values, the mean value for the obese mice being 331 and for the lean ones 311 mg/100 ml The unexpected hyperglycemia of the lean litter mates might have been induced merely by the injection procedure However as shown in Fig 2 the decline of serum glucose was significantly less in the obese group With the exception of the sample taken after 30 min, there was no difference in the rate of disappearance of insulin from the serum of the obese and the lean mice The semilogarithmic plot of insulin concen

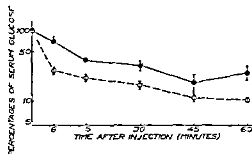


Fig 1

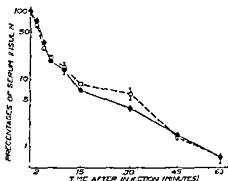


Fig 2

Fig 1 The decline in serum insulin following injection of 600 U insulin/kg b w into obese (●) and lean (○) mice Percentages of insulin concentration at zero time, mean values ±S E M plotted on vertical axis Between 3 and 9 observations in each point

Fig 2 The fall in serum glucose following injection of 600 U insulin/kg b w into obese (● and lean (○) mice Percentages of serum glucose concentration at zero time mean values ±S E M plotted on vertical axis Five observations in each point

trations versus time, shows that there was not a continuous exponential fall The data might, however, be consistent with two overall exponential processes intersecting at about 6 min after injection.

The results obtained after the administration of the tracer insulin dose are presented in Fig 3 The figures are calculated from the amount of radioactivity bound by insulin antibodies per aliquot of mouse serum at the stated times, the zero time value for each animal is equal to 100 per cent A comparison of the percentage fall in radioactive serum insulin with time between the obese and lean mice did not reveal any conclusive difference between the two groups of animals

However their different average serum insulin concentrations which in the untreated obese mice amounted to 247 ± 16 mμg/ml and in the lean controls to 8 ± 1 mμg/ml indicates a considerably greater absolute turnover rate for the obese animals

TABLE I Serum insulin content (μg ml) at representative intervals following injection of 600 U insulin/kg b w into obese and lean mice The number of animals in each group is indicated within brackets Mean values ± S E M

Animals	Time after insulin injection (min)								
	0	2	4	6	10	15	30	45	60
Obese	371 ± 24 (9)	277 ± 37 (4)	132 ± 21 (4)	70 ± 5 (9)	52 ± 4 (4)	24 ± 2 (5)	13 6 ± 0 9 (9)	5 5 ± 0 2 (5)	2 5 ± 0 3 (5)
Lean	371 ± 18 (9)	233 ± 32 (5)	105 ± 13 (5)	77 ± 7 (9)	53 ± 4 (4)	34 (3)	22 7 ± 0 4 (9)	5 3 ± 0 3 (4)	2 7 ± 0 6 (5)

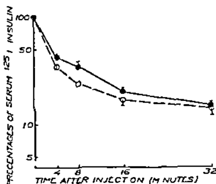


Fig 3 The decline in serum labelled insulin following injection of a tracer dose ^{125}I labelled insulin into obese (●) and lean (○) mice Percentages of labelled insulin concentration at zero time mean values ±S E.M plotted on vertical axis Between 3 and 5 observations in each point

Discussion

The technique of binding the insulin to antibodies when measuring the disappearance of the hormone from serum as used in the present study obviated some of the problems of earlier studies where trichloroacetic acid precipitable radioactivity was used as a measure of intact insulin (Scott *et al* 1958) The possibility that the labelled insulin used might be resistant to destruction is not likely to be relevant in the present study (Izzo *et al* 1967) A similar preparation of iodinated insulin used in the earlier in vitro experiments on homogenates was found to be destroyed to the same extent as the non labelled carrier insulin (Westman 1968b)

There was an initial rapid phase of the disappearance of insulin from serum as previously reported by others (Izzo *et al* 1967 McAdams Knox and Wilcox 1967) Many processes contribute to the disappearance of endogenous and exogenous serum insulin The relative importance of processes such as diffusion into extravascular fluids adsorption to cell surfaces destruction and excretion probably varies at different times after the injection and with the load The first rapid decline in hormone concentration might be dominated by diffusion of intravascular insulin into interstitial fluid (Rasio *et al* 1967) Furthermore high levels of circulating insulin might exceed the renal threshold and insulin would then be excreted by the kidney These considerations explain the deviation from linearity in the curves Linear semilogarithmic plots would only be expected if insulin removal from plasma in both instances was determined by a single process whose rate was mainly dependent on the instantaneous concentration of insulin in plasma (*cf* Izzo *et al* 1967) Furthermore whereas the disappearance of tracer labelled insulin reflects insulin removal from a pool of constant and physiological magnitude the decline of insulin concentration following a big dose exogenous insulin reflects insulin removal from a varying and enlarged pool It is not expected that decay constants would be similar for such different experimental situations and in fact the shapes of the two curves were different

The rates of decline of serum insulin concentration following large doses of insulin did not differ significantly between obese and lean animals No support was thus found for the view that an increased serum insulin disappearance rate contributes to the marked resistance displayed by obese mice to the hypoglycemic effect of massive doses of exogenous insulin (Westman 1968a) However, when measured under more physiological circumstances after administration of a tracer dose, the absolute disappearance rate of insulin from the sera of the obese animals was considerably higher

The increased turnover of serum insulin in obese animals is in accordance with the morphological evidence for a high rate of insulin secretion from the B-cells of obese mice (Gepts Christophe and Mayer 1960, Hellman and Petersson 1960) Both the pool size and the inward and outward fluxes of serum endogenous insulin are thus increased in obese animals This conclusion need not conflict with apparently similar disappearance rates of serum insulin following very large doses of exogenous hormone In the latter case it could be assumed that insulin efflux from serum would be determined by initial insulin concentrations which were equal in both groups This assumption implies either that the mechanisms for insulin disposal were operating below maximal capacity in both groups of animals (Samols and Ryder 1961, Burgi *et al* 1963) or that their maximal capacities for insulin disposal were in fact equal

These experiments leave undecided the participation in serum insulin disposal of the insulin degrading system in adipose tissue homogenates (Westman 1968b) The *access of circulating insulin to the system in intact cells is in any case uncertain*

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From the Department of Pharmacology, University of Helsinki Finland

The Effect of Some Substances on the Isolated Bull Retractor Penis Muscle

By

Erik Klinge

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Abstract

Klinge, E *The effect of some substances on the isolated bull retractor penis muscle* Acta physiol scand 1970 78 280—288

The effect of catechol amines, other biogenic amines and polypeptides acetylcholine and other choline esters nicotine, and various autonomic blocking agents on strips of the bull retractor penis
conc
and
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at interruption of the adrenergic activity by impulses conducted mainly along the pelvic nerves
e pelvic nerves probably form cholinergic ganglionic or postganglionic inhibitory synapses
th the adrenergic nerves concerned The peripheral autonomic mechanism of penile erection
discussed on the presumption that the muscles of the penile artery are subjected to a similar
ervous control as the retractor penis It is assumed that there are no differences in principle
etween bulls and dogs with respect to the nervous mechanisms in question

The dog retractor penis muscle contracts on stimulation of the lumbar sympathetic chain and relaxes on stimulation of the pelvic nerves (Langley and Anderson 1895) The bull retractor penis contains a dense adrenergic innervation (Klinge Pohto and Solatunturi 1970 Klinge 1970a) and acetylcholine also is present in the muscle (Klinge 1970b) But it still is obscure what happens in the retractor penis of any mammalian species upon stimulation of the pelvic nerves i e what substance is the nervous transmitter of its relaxation In the present study an attempt is made to approach this problem by examining the action exerted by various compounds directly on the cells of the bull retractor penis A preliminary report of some of the results presented here has been given elsewhere (Klinge 1967, 1969b)

Material and methods

Preparations The bulls were of the Ayrshire breed and weighed 150—250 kg Immediately after slaughtering the retractor muscles were removed and transported to the laboratory in Tyrode solution at 35° C Longitudinal strips of about 4 cm were usually cut from the distal

final concentration of the respective base in the organ bath Before beginning of the actual experiments the reactivity of each strip was tested with one or two dose response curves to adrenaline During a stimulation period of 6 to 8 hrs the sensitivity of the muscle remained unchanged or showed some increase The blocking agents when used, were continuously present in the bath fluid The number of strips examined was 170

List of drugs used l adrenaline d bitartrate (C H Boehringer Sohn), l noradrenaline-d hydrogentartrate (Fluka AG), isoprenaline sulphate (Siegfried SA), tyramine hydrochloride (Sigma Chemical Company), dopamine (3 hydroxytyramine hydrochloride, Sigma Chemical Company), 5 hydroxytryptamine creatinine sulphate (Fluka AG), histamine acid phosphate (British Drug Houses Ltd), acetylcholine chloride (Sigma Chemical Company), propionylcholine iodide (Fluka AG), butyrylcholine iodide (Fluka AG), methacholine (d l acetyl β methylcholine [illegible] chloride (Fluka AG), bethanechol [illegible] gen tartrate (British Drug Houses [illegible] [illegible]cinon®, Sandoz AG), vasopressin ([illegible] , Ciba)

Blocking a[illegible] French Laboratories), chlor[illegible] Baker Ltd), yohimbine hydrochloride (E Merck AG), [illegible]ne sulphate (Ph Nordica), methysergide (Deseril® [illegible] Star Ab), physostigmine salicylate (Ph Fennica VII),

Results

Noradrenaline Low concentrations of noradrenaline as a rule produced vigorous dose dependent contractions in the muscle strips (Fig 1) Signs of desensitization to noradrenaline in the course of an experiment were never observed In the continuous presence of noradrenaline in the bath fluid (*e g*, 100 ng/ml) the same almost maximal degree of contraction was maintained for several hours The excitatory effect of noradrenaline was highly sensitive to *phenoxybenzamine, i e*, in the presence of 1 ng/ml of this *α* adrenergic blocking agent a 10 fold concentration of noradrenaline was required to produce the same contraction as before the blockade (Fig 2) The *α* adrenergic blocking effect exhibited by *chlorpromazine* was on weight basis only about 10 times weaker than that of phenoxybenzamine, whereas that of *yohimbine* was about 1000 times weaker

Adrenaline The contracting power exerted by adrenaline was the strongest of all the amines examined, being about three times as strong as that of noradrenaline (Fig 1) The adrenaline induced contractions showed the same rapid onset and were blocked by phenoxybenzamine chlorpromazine and yohimbine in the same ratio as the contractions brought about by noradrenaline In the presence of an *α*-adrenergic blocking agent the muscle strips were relaxed by adrenaline only when kept in a contracted state by another substance Bradykinin proved a suitable contractor for the demonstration of adrenaline-induced relaxations although the maintenance of a constant basic level of contraction was not quite successful (Fig 3),

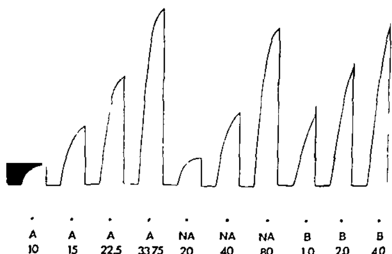


Fig 1 Isotonic contractions of the isolated bull retractor penis muscle induced by adrenaline (A) noradrenaline (NA) and bradykinin (B) Doses refer to the final concentration of the corresponding base or peptide in the organ bath in ng/ml The bradykinin induced contractions show a slower onset and progress than those brought about by the amines

The relaxations showed dose dependence and they were produced by as low concentrations of adrenaline as the excitatory responses in the uncontracted muscle

Isoprenaline Isoprenaline regularly relaxed the strips contracted by any other substance But it usually did not produce additional relaxation in the uncontracted muscle The situation presented in Fig 4 therefore is considered exceptional Likewise the spontaneous activity seen in the same figure occurred very seldom In the presence of 100 ng/ml of *pronethalol* the inhibitory effect of isoprenaline was reduced by 70 % approximately

Other amines The contracting potency of *tyramine* was about one tenth of that of adrenaline The contractions produced by this amine showed a considerably slower onset than those brought about by adrenaline (Fig 5) or noradrenaline The effect of *dopamine* was identical with that of tyramine with the exception that its contracting power was slightly weaker The contractions produced by tyramine and dopamine were similarly affected by phenoxybenzamine and chlorpromazine as were the noradrenaline induced contractions

The contracting capacities of *histamine* and *5 hydroxytryptamine* were on weight basis of the same order of magnitude as those of tyramine and dopamine and they were abolished by mepyramine (100 ng/ml) and methysergide (1 μg/ml), respectively, but remained unaffected by a phenoxybenzamine concentration of 1 ng/ml The contractions brought about by histamine or 5 hydroxytryptamine were strictly dose-dependent like all responses to any of the amines examined in the present study Neither histamine nor 5 hydroxytryptamine relaxed the strips contracted by noradrenaline

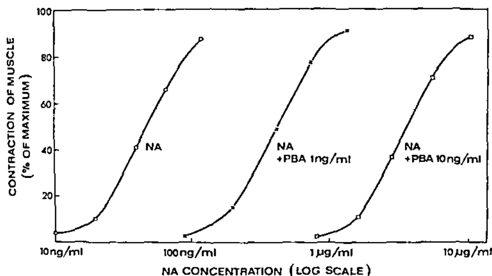
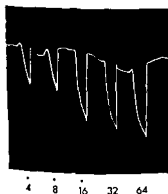
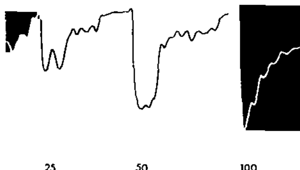


Fig 2 Contraction of the retractor muscle induced by noradrenaline alone (NA) and by noradrenaline in the presence of various concentrations of phenoxybenzamine (PBA)

Acetylcholine Most preparations were contracted by acetylcholine only when the concentration exceeded 10 µg/ml, but in several instances no response was obtained even to 100 µg/ml The contractions of the strip that exhibited the highest sensitivity to acetylcholine are illustrated in Fig 6 There never was a linear dose-response relationship The muscle commonly commenced to relax before washing was begun A physostigmine concentration of 1 µg/ml did not intensify the contractions that

4 8 16 32 64 25 50 100

Fig 3 Fig 4

Fig 3 Relaxation of the muscle induced by various concentrations (ng/ml) of adrenaline The strips were kept in a contracted state with bradykinin (100 ng/ml) and the bath fluid further contained phenoxybenzamine 1 µg/ml

Fig 4 Relaxation of the retractor muscle induced by various concentrations (ng/ml) of isoprenaline

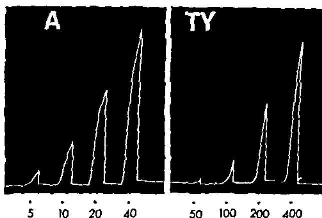


Fig 5 Contractions induced in the same strip of retractor penis muscle by various concentrations (ng/ml) of adrenaline (A) and tyramine (TY) The tyramine-induced contractions show a slower onset than those produced by adrenaline

were unaffected also by high concentrations of hexamethonium (100 μg/ml) and atropine (1 μg/ml) An atropine concentration of 10 μg/ml slightly reduced the contractions and a phenoxybenzamine concentration of 1 μg/ml fully inhibited them In strips continuously contracted by noradrenaline (100 ng/ml) acetylcholine produced feeble and irregular movements that remained uninfluenced by physostigmine and atropine Dose-dependent relaxations were not observed

Other choline esters The experiments with propionylcholine, butyrylcholine methacholine, carbamylcholine and bethanechol yielded the same results as those with acetylcholine The only difference in principle was that with these choline esters dose-dependent contractions could be obtained But in all cases the threshold concentration was at least 10 μg/ml In the contracted muscle propionylcholine and butyrylcholine produced more intense irregular movements than the other choline esters but no dose-dependent relaxations

Nicotine The muscle strips were completely unaffected by nicotine in concentrations lower than 100 μg/ml With gradually increasing concentrations quite feeble

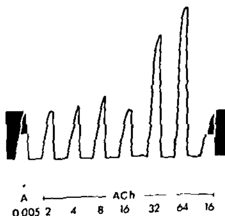


Fig 6 Contractions of the retractor muscle induced by adrenaline (A) and acetylcholine (ACh) The concentrations of the bases in the bath are given in μg/ml The responses to acetylcholine do not exhibit a linear dose response relationship

contractions were obtained until 2 mg/ml was reached. Usually at this concentration a maximal contraction abruptly broke off. In spite of repeated washings no relaxation occurred until large doses of isoprenaline were applied. The nicotine-induced contractions were unaffected by hexamethonium (100 µg/ml) or atropine (1 µg/ml) but they were considerably reduced or fully inhibited by the phenoxybenzamine concentration of 1 µg/ml.

Posterior pituitary hormones. No contractions were effected by even high concentrations (0.5 I.U./ml) of *vasopressin* or *oxytocin*. But the strips contracted by noradrenaline (100 ng/ml) were regularly relaxed by high concentrations of vasopressin. The lowest effective concentration usually was 0.05 I.U./ml. A linear dose-response relationship was not constantly observed. Oxytocin showed a similar relaxing effect though it commonly proved somewhat weaker.

Bradykinin was by far the most potent contractor of all the compounds examined (Fig. 1). In most preparations considerable contraction was effected by the concentration of 0.2 ng/ml and a linear dose-response relationship always existed. The bradykinin induced contractions were uninfluenced by all blocking agents used in this study. There were no responses to *angiotensin* (50 µg/ml) either in the relaxed or in the contracted muscle strips.

Discussion

Contraction of the retractor penis muscle. The isolated bull retractor penis is highly sensitive to noradrenaline. The manner in which the excitatory effect of noradrenaline is inhibited by phenoxybenzamine (Fig. 2) fulfils one of the basic criteria established for competitive α adrenergic blockade (Birmingham and Szolcsanyi 1965). The pharmacological data support the view that the tonic contraction of the bull retractor penis is maintained by a continuous discharge of adrenergic nerve impulses. Some indirect evidence for the validity of this conception probably is provided by the early work of Brucke and Oinuma (1910). In the contracted dog retractor penis they registered continuous waves of action potentials that were strengthened on sympathetic stimulation. It is conceivable that the concentration of noradrenaline at the receptor sites is so high that inhibition of the muscle cannot be achieved with pharmacological doses of any α adrenergic blocking agent. The low or absent catechol O methyl transferase activity in the muscle (Klinge 1970c) may to some extent impair the inactivation of released noradrenaline.

It is unlikely that adrenaline as a local substance would be involved in maintaining the contraction of the muscle since there probably is no adrenaline in it (Klinge 1970a). Injected adrenaline in dogs invariably increases the tone of the retractor penis (e.g. Elliott 1905, Dale 1906) but circulating adrenaline hardly exerts a continuous excitatory action directly on the muscle cells. The effect of tyramine and dopamine on the isolated bull retractor penis should be mediated at least in part by the release of noradrenaline. This assumption is based on the slow onset of action and on its high susceptibility to α adrenergic blockade. Bradykinin [illegible] enormous potency in contracting the muscle strips. [illegible]

say of bradykinin is considered elsewhere (Klinge 1969b) So far there is no evidence to assume that bradykinin might take part in the physiological regulation of the tonus of the muscle The same statement can be applied to histamine 5 hydroxytryptamine and angiotensin

Relaxation of the retractor penis muscle The present results suggest but clearly do not establish that the bull retractor penis is supplied by a single type of efferent nerve fibres, *i e* adrenergic fibres mediating impulses for the tonic contraction If this hypothesis is correct it is logical that the discharge of adrenergic impulses has to be interrupted to obtain a relaxation of the muscle There probably are inhibitory synapses between the pelvic nerves and the adrenergic nerves in question Some indirect support for this assumption might again be found in the investigations of Brucke and Oinuma (1910) They noted that stimulation of the pelvic nerves abolishes the action potentials in the dog retractor penis and simultaneously relaxes the muscle

According to the present results it is unlikely that acetylcholine or some other choline ester exerts an inhibitory effect directly on the muscle cells *i e* there are no muscarinic receptors that would mediate an acetylcholine induced relaxation The bull retractor contains considerable amounts of acetylcholine and it is not excluded that acetylcholine plays some role in the function of the sympathetic nerves running in the muscle (Klinge 1970b) It has been suggested that in the adrenergic nerve terminals in the dog retractor the release of noradrenaline is mediated by acetylcholine (Armitage and Burn 1967) The effect of nicotine on the isolated bull retractor penis is similar to its effect on strips of the dog retractor described by Edmunds (1920) The present results indicate the absence of ganglionic synapses in the bull retractor This is compatible with histochemical observations (Klinge Pohto and Solatunturi 1970) It is not known whether the nicotine induced maximal contraction is due to nicotine itself or to a release of noradrenaline

The effects of isoprenaline and adrenaline well establish the existence of β adrenergic receptors in the bull retractor So far there is however no support for the assumption that stimulation of such receptors by adrenaline would be of any significance for the physiological relaxation of the muscle The bull retractor probably is devoid of adrenaline and dichlorisoprenaline completely fails to inhibit the relaxation of the dog retractor effected by stimulation of the pelvic nerves (Luduena and Grigas 1966) In the experiments of Dale (1906) i v injection of adrenaline did not relax the retractor of dogs treated with ergot

In this study the posterior pituitary hormones were the only physiological substances that relaxed the muscle strips contracted by noradrenaline With large doses this effect was constant but not always dose dependent Further investigations are required to evaluate whether either of these hormones or some other peptide is involved in the physiological relaxation of the muscle The possibility also must be considered that there is no need for a substance that would exert a direct inhibitory action on the muscle cells the presumable interruption of the nervous impulses alone being enough

Peripheral autonomic mechanism of penile erection The experiments of Langley and Anderson (1895) have shown that in dogs and cats lumbar sympathetic stimulation causes contraction of the retractor penis and simultaneous constriction of the penile blood vessels and that stimulation of the pelvic nerves reduces the tonus of the retractor and simultaneously induces penile erection Several authors have suggested that decreased arterial resistance is the most essential condition for penile tumescence (*e g* Kolliker 1852 Dorr and Brody 1967 for further literature see Muller and Dahl 1912 Gruber 1933 Klinge 1969a) It is conceivable that a continuous constriction of the penile artery restricts the entrance of blood into the cavernous bodies during the relaxed state of the penis and that there is close similarity in the nervous regulation of the tonus of the retractor and of the muscles in the wall of the penile artery The considerable thickness of the muscular layers of the human penile artery has been emphasized by Kiss (1921) The bull penile artery also is thick walled and contains a dense adrenergic innervation (Klinge 1969c) At perfusion its responses to drugs (Penttila 1966 Penttila and Klinge 1966) closely resemble those of the isolated bull retractor penis

The penile erection evoked in dogs by stimulation of the pelvic nerves is intensified by physostigmine (Henderson and Roepke 1933 Bacq 1935) and partially reduced by atropine but fully inhibited by hexamethonium (Dorr and Brody 1967) The last mentioned authors failed to observe any sign of erection at intraarterial administration of even enormous doses of acetylcholine These data suggest that there are cholinergic ganglionic or postganglionic inhibitory synapses between the pelvic nerves and the sympathetic nerves concerned The existence of inhibitory synapses between those nerves has been considered a century ago (*e g* Loven 1866 Goltz and Freusberg 1874) According to Cannon (1914) circulating adrenaline strongly prevents erection This is consistent with the observation that i v injection of this amine causes constriction of the penile blood vessels (Elliott 1905 Dale 1906) It has been reported that i v administration of either of the posterior pituitary hormones to dogs results in slight swelling of the penile erectile bodies (Holmquist and Olin 1969) This is not in contradiction to the results of the present study But it remains to be solved however whether vasopressin or oxytocin or some other peptide is involved in the physiological erection of the penis

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Unit Responses in the Cochlear Nucleus of the Rat to Noise and Tones

By

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Abstract

MØLLER, AAGE R. *Unit responses in the cochlear nucleus of the rat to noise and tones.* Acta physiol. scand. 1970. 78. 289—298.

The spectral integration of single units in the rat's cochlear nucleus has been investigated by two different methods, namely:

1. by measuring the difference in sensitivity to broad band noise and to pure tones of a frequency equal to the unit's characteristic frequency (CF).

2. [illegible]

indicate that the spectral resolution of single units in the rat cochlear nucleus above threshold is higher for broad band noise than the measurements with pure tones indicate (tuning curves). The time patterns of the neural responses to noise and tone stimulation, judged from a dot display of the discharges, show no apparent difference except for units with a very low CF.

It is wellknown that the response areas of single auditory nerve fibers differ considerably from the mechanical filter characteristic of the basilar membrane. Thus, the neural response areas for pure tones (tuning curves) are much narrower than implied by the characteristics of the basilar membrane. This sharpening of frequency selectivity is probably accomplished by interaction between neighbouring receptors and/or nerve elements. The inhibition which has been shown both in primary fibers (Nomoto *et al.* 1964, Sachs and Kiang 1968) and in the cochlear nucleus (Galambos and Davis 1943, Moushegian *et al.* 1962, Greenwood and Maruyama 1965) may very well play an important role in the sharpening of the tuning curves. The response area of units of the eighth nerve and in the cochlear nucleus has been shown to have an inhibitory area on both the high and the low frequency side. These properties have been determined by single pure tones which were used to map excitatory and inhibitory areas.

The effect of these inhibitory areas, as well as of the nonlinear characteristics of the excitatory process in the cochlea, may not be represented in the tuning curves which are based on the result of threshold measurements using discrete pure tones to the same degree as is the case for stimulation with broad band noise Since the sounds which the auditory system analyses have a broad spectral distribution it is of interest to study the spectral resolution of the auditory system with regard to broad band signals In the present investigation the spectral integration of broad band noise by single units in the cochlear nucleus of the rat has been estimated by determining the difference in their responses to pure tones and to broad band noise Such values were obtained at threshold and 10 dB above threshold

The objective of the present study requires that certain basic information be set forth about the action of spectral filters The amount of noise energy transmitted by a linear bandpass filter is proportional to its bandwidth while the transmittance of a sinusoidal signal with a frequency equal to the center frequency of the bandpass filter is independent of the bandwidth The ratio (or the difference in dB) between the transmitted noise and the sinewave signal can thus be used as a measure of a filters bandwidth If the single units had the same properties as linear filters bandwidth values obtained from tuning curves (at 3 dB above the point of lowest threshold) and from the difference in sensitivity to noise and tones would have been equal Since the sharpening of the tuning curves in contrast to the selectivity of the basilar membrane is non linear the spectral resolution of the auditory system in response to signals with a wide spectral distribution may not be equal to the width of the tuning curves

The aim of the present study is to compare the width of the pure tone response areas (tuning curves) of single units in the cochlear nucleus with the degree of spectral integration of broad band noise The experiments were performed on rats and the difference in sensitivity to noise and to pure tones with a frequency equal to the units CF was used as a measure of the bandwidth of the single units These values of bandwidth were then compared with those obtained by measuring the width of the tuning curves

Methods

The same methods as described previously (Moller 1969) were used in the present study Extracellular recordings were obtained from single units in the rat cochlear nucleus The stimuli consisted of 50 msec sound bursts presented at a rate of 6/sec The stimulus response curves were based on the responses to 32 such sound bursts The number of dischargers were counted during the time the stimulus was on and the average number of discharges was calculated and plotted as a function of sound intensity

Tuning curves were constructed from threshold values at different tone frequencies The sound intensity was changed in 5 dB steps and the tone frequency was varied in order to find the frequency which gave a just noticeable increase in the firing rate

Special consideration was paid to the intensity and spectral distribution of the noise The level of intensity and the spectrum of the noise were measured at the input of the condenser microphone which was used as a sound source A Radiometer wave analyzer type Frq 1 was used at a bandwidth setting of 2 Hz to measure the spectral distribution of the noise The energy in a 1 Hz band (spectrum level) was calculated on the basis of the measurements The relation between intensity of noise and tone stimulation was checked frequently during the experiments and the variations did not exceed ±0 5 dB

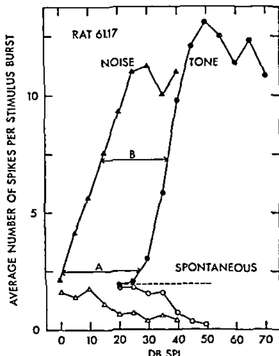


Fig 1 Stimulus response curves of a typical unit to noise and tone stimulation The average number of discharges evoked during stimulation with 32 sound bursts of 50 msec duration is shown as a function of sound intensity in dB re 0 0002 μB (SPL) The intensity of the noise refers to the energy in a 1 Hz band The distance A gives the difference in sensitivity to noise and tone at threshold and the distance B gives the difference at an average firing level which is 10 dB above threshold for the tone

Results

The present paper is based on the analysis of the response of 75 units in 27 animals These units were selected on the basis of their response to pure tones Only those units were included which gave a sustained response to pure tones and had a response area (tuning curve) for pure tones of the common type *i e*, with an abrupt rise in threshold when the tone frequency was increased above the units' characteristic frequency (CF) (*cf* Moller 1969) All such units except two could be excited by broad band noise These two units had a low CF (700 and 940 Hz) Broad band noise also inhibited the spontaneous activity of these two units to a degree which was dependent on the intensity of the noise

The difference (in dB) in threshold to broad band noise and pure tones with a frequency equal to the units CF was determined from stimulus response curves in the way shown in Fig 1 In this figure the filled triangles represent the average number of discharges evoked by 32 bursts of noise (duration 50 msec) as a function of the sound level in a 1 Hz broad band The filled circles show, in a similar way the response to a pure tone whose frequency is equal to the unit's CF The open circles show the average spike count during the first 50 msec following the end of the tone bursts The dashed line indicates the level of the unit's spontaneous activity The distance A in Fig 1 represents the difference in threshold of tone and noise and the distance B represents the difference in sensitivity measured 10 dB above the threshold

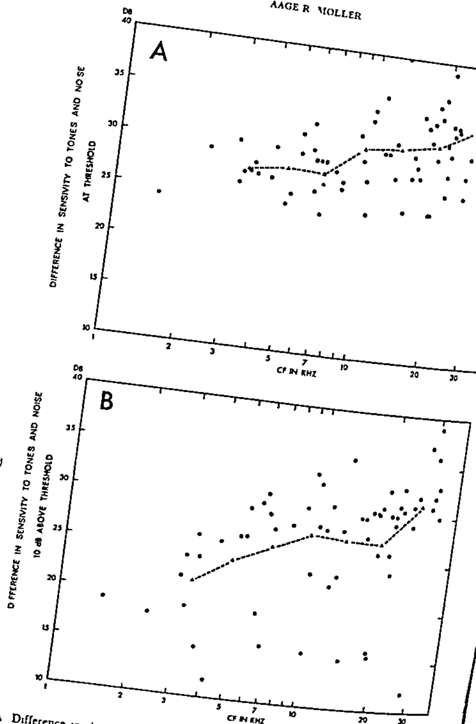


Fig 2A Difference in threshold for stimulation with pure tones at CF and broad band noise as a function of the CF

B Difference in the sound level required to evoke the same average firing rate as that produced by a pure tone 10 dB above the unit's threshold The difference is based on the spectrum level of the noise (energy in a 1 Hz band) in both graphs Mean values calculated over half octaves are shown by triangles connected by dashed lines

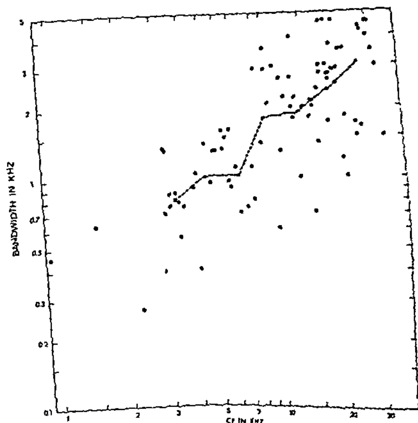


Fig 3 Scatterdiagram showing the width of tuning curves measured 10 dB above the threshold at CF as a function of CF The mean values (in half octave bands) are shown by triangles connected by dashed lines

for the tone Threshold is here defined as the sound intensity which increases the spontaneous firing rate 20 % or in case of no spontaneous activity an average firing of 0 1 discharge per sound burst Fig 2 A shows the difference in threshold to noise and tones measured in the way as illustrated in Fig 1 Fig 2 B shows, in a similar way, the difference in sensitivity measured 10 dB above threshold (distance B in Fig 1) These latter values show a larger scatter than the difference in threshold

In Fig 2 the triangles connected with dashed lines give the mean values determined in half octave bands From this it is seen that the difference in sensitivity to tones and noise increases slightly with the CF of the units The values obtained 10 dB above threshold (Fig 2 B are slightly lower than those based on threshold values The difference in threshold to tones and noise of most units falls between [illegible] and [illegible] dB That means that the noise energy referred to a 1 Hz broad band is 300 to [illegible] times more efficient in stimulating these units than a pure tone whose frequency is equal to the units CF When the rat's ear is stimulated [illegible] band noise the

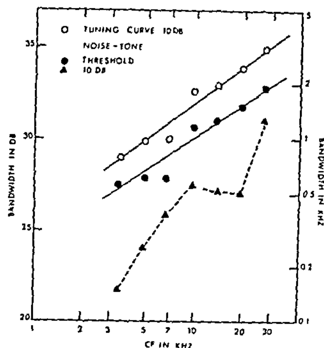


Fig. 4. Comparison of the results shown in Fig. 2 and 3. The mean values of bandwidth measured from tuning curves (Fig. 3) are shown together with the mean values of the difference in sensitivity to noise and tones from Fig. 2. The bandwidth is given in dB and in kHz as a function of the units' CF.

spectral energy is thus integrated over a frequency range which varies between 300 and 3000 Hz for different units. Should the units have the same properties as linear filters (such as mechanical and electrical filters composed of linear elements) their bandwidth could equally well have been estimated from the tuning curves or from the difference in the sensitivity to pure tones and white noise and the same value of bandwidth would result. Since the units cannot be assumed to have the same properties as linear filters, it is of interest to compare the width of the tuning curves with the bandwidth values obtained by noise measurements.

The width of the tuning curves of single units in the cat's cochlear nucleus has been determined earlier (Kiang 1965). The bandwidth of units with a high CF was shown to be larger than that of units with low CF. Similar results were obtained in the rat in the present study as illustrated in Fig. 3 which also shows the mean values determined in half octave bands (triangles and dashed lines). The measurements were made 10 dB above the units' threshold in the same way as that of Kiang (1965). The units' threshold was estimated from stimulus response curves as illustrated in Fig. 1. The present results are similar to those of Kiang and the bandwidth of units with a high CF is larger than those with a low CF. Fig. 4 shows a comparison of the mean bandwidth determined from the difference in threshold to noise and pure tones (filled circles) and that measured on tuning curves (open circles). The bandwidth is given in dB (10 log bandwidth) and kHz. It is seen that the bandwidth measured in the two different ways increases with the units' CF at almost the same rate, but the bandwidth based on noise measurements is only about half of the bandwidth values based on the tuning curves.

In comparing absolute values of bandwidth, however, it must be kept in mind that the width of the tuning curves was determined 10 dB above the lowest threshold and such values are therefore not directly comparable to bandwidth values obtained in noise measurements To be comparable the width of the tuning curves should be measured 3 dB above the point of lowest threshold Unfortunately such measurements cannot be performed on tuning curves with sufficient accuracy Iso rate curves (*i e* curves which relate that given sound intensity which produces a certain firing rate as a function of tone frequency *cf* Møller 1969) which can be determined with much greater confidence than tuning curves, revealed that the units' response areas were nearly twice as broad 10 dB above the lowest threshold as compared to the response area measured 3 dB above With the measured bandwidth of the tuning curves reduced by a factor of two, the mean bandwidth values determined by comparing noise and tone threshold thus agreed very well with those based on tuning curves

The bandwidth values based on measurements of the difference in sensitivity to tones and noise at a level which is 10 dB above the threshold of the tone (*cf* Fig 1) are in most cases smaller than those based on the measurements at the threshold (Fig 2 B) The reason for this difference is that the slope of the stimulus intensity curves for noise is smaller than for pure tones so that distance B (in Fig 1) becomes smaller than distance A The mean values of the difference in sensitivity to noise and tones 10 dB above threshold (from Fig 2 B) are also plotted in Fig 4 (triangles) and those values are in the entire response range significantly smaller than those representing the difference in noise and tone threshold These results indicate that the spectral resolution (with regard to broad band signals) is greater *above* threshold than near threshold The slope of the stimulus response curves for noise can in rare cases be greater than that for pure tones Furthermore, in many cases the maximal obtainable firing rate is greater following stimulation with pure tones than it is after noise stimulation

The results shown thus far were based on the mean discharge rate during stimulation In order to see whether the temporal pattern of the unit s response was different for noise and tone stimulation the time pattern of the response of 64 units to noise and tones were compared by examining records such as those shown in Fig 5 In these records each individual nervous discharge is indicated by a dot Each horizontal line of dots depicts the spike discharges following one stimulus presentation with time proceeding from left to right and starting at the initiation of each 50 msec long sound burst The latter point is shown as a faint dot at the extreme left of each line Sound intensity in dB is indicated by legend numbers It is seen that the number of discharges evoked by noise and tones are different in accordance with the results shown in Fig 3 However the time pattern of the discharges does not show any apparent difference between tone and noise 22 of the 65 units investigated in that way showed a semiperiodic time pattern exemplified in Fig 5 while others (42) showed an apparent random distribution of discharges Examination of records similar to those in Fig 5 revealed no difference in the time pattern of the responses to noise and pure tones in 63 of 65 units investigated The two units which showe

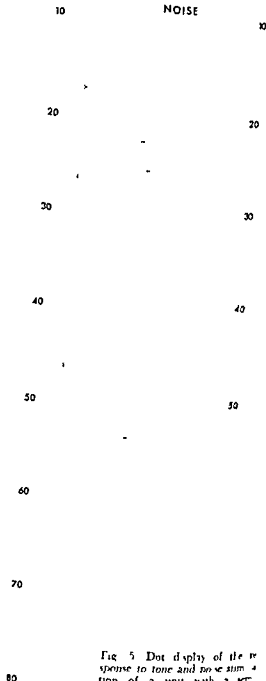


Fig. 5. Dot display of the response to tone and noise stimulation of a unit with a semi-periodic firing pattern to stimulation. The intensity is given in dB SPL by legend numbers. For the noise the intensity refers to the energy in a 1 Hz band. Duration of stimulation was 50 msec. The first dot in each row marks the initiation of the stimulus.

difference had a low CF and contrary to all other units studied, their spontaneous activity was inhibited when stimulated with noise although tone stimulation increased the firing rate in the usual way

Discussion

The width of the tuning curves and the spectral integration are both measures of the spectral resolution and the term "bandwidth" is used in this paper for both these two measures The present study shows that the average width of the tuning curves (at 3 dB points) of single units in the rat cochlear nucleus provides values which are in good agreement with the frequency range over which wide band noise near threshold is integrated The values of bandwidth increase with the units' CF Spectral integration of noise 10 dB above the threshold is smaller than it is at threshold All units studied had a tuning curve of the common type with a steep high frequency skirt (Møller 1969)

It has been shown earlier that the tuning curves of primary units in the monkey's and the cat's eighth nerve (Nomoto *et al* 1964, Sachs and Kiang 1968) as well as those of the cochlear nucleus are surrounded by inhibitory areas (Greenwood and Maruyama 1965) The results of the present study indicate that these inhibitory areas do not modify the excitatory response very much near threshold but, above threshold the excitatory area is depressed by the simultaneous stimulation of the inhibitory areas The interaction would result in a smaller width of the unit's response area and this would appear when sounds with a broad spectrum were used as stimuli It is thus probable that spectral resolution in units of the auditory periphery depends on the type of sound presented

The finding that low frequency units are not excited by wide band noise agrees with the results of Greenwood and Maruyama (1965) These workers found that while a neuron responded to narrow band noise centered at its CF response might cease when the bandwidth of the noise was increased to cover also the inhibitory areas of the unit

Although the output of the noise generator used in the present investigation was uniform the excitation at the level of the cochlear was not likely to be uniform of several reasons The sound generating system and the middle ear coloured the noise (Møller 1969) It is not clear whether a nonuniform sound spectrum influences the results The frequency range which is covered by each unit's excitatory and inhibitory areas is however rather small and the noise spectrum may be regarded as almost uniform within this range

The bandwidth values estimated from mechanical measurements on the basilar membrane are considerably larger than the above mentioned values. For many years only values from the relatively low frequency region of the basilar membrane in human cadaver ears were available (v Bekesy 1960) but lately Johnstone and Boyle (1967) obtained a few measurements in another region of the basilar membrane (cat) which responds to much higher frequencies (19 kHz Resonance curves of

RAT 6710 CF 6 2 KHZ

TONE

NOISE

10

20

30

40

50

60

70

80

STIMULUS

Fig 5 Dot display of the response to tone and noise stimulation of a unit with a semiperiodic firing pattern to stimulation. The intensity is given in dB SPL by legend numbers. For the noise the intensity refers to the energy in a 1 Hz band. Duration of stimulation was 50 msec. The first dot in each row marks the initiation of the stimulus.

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Studies of the Damped Oscillatory Response of the Auditory Frequency Analyzer

By

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Abstract

MØLLER AAGE R *Studies of the damped oscillatory response of the auditory frequency analyzer* Acta physiol scand 1970 *78* 299—314

The temporal characteristics (system impulse response) of the peripheral auditory spectrum analyzer, up to the level of the neural excitation has been studied This was accomplished by recording the average firing rate of single units in the rat cochlear nucleus in response to paired clicks in which the interval between clicks was varied over a large range The results show that the system impulse response of all units studied was a damped oscillation The frequency of the oscillation was equal to the characteristic frequency of the unit On basis of these recorded responses the auto-correlation of the system impulse response was computed It was furthermore found that bandwidth values determined on the basis of the derived auto-[illegible]

When the ear is stimulated with pure tones the basilar membrane will vibrate with a larger amplitude within a narrow region Various narrow segments along the membrane are tuned to different frequencies Consequently, each region exhibits a resonant action with regard to a certain tone frequency As is the case for other resonant systems it is generally accepted that each point on the membrane exhibits a damped oscillatory response to excitation by brief sounds such as clicks (The response of a system to excitation with a short impulse is called the system impulse response and it completely characterizes a linear system) *Direct* determination of the system impulse response of the basilar membrane by observing its response to excitation with a short pulse has not been made earlier presumably due to the great technical difficulties of such a measurement

v Békésy and Johnstone and Boyle show bandwidth values (at 3 dB points) which are about three times larger than those of the tuning curves of the rat (at 3 dB points)

The frequency selectivity of the basilar membrane as well as that of the tuning curve of a single unit has often been expressed as the ratio between the frequency of highest response (CF) and the bandwidth This ratio is sometimes named Q a name which has its origin in the theory of single resonators It might be more descriptive instead to use the term *relative bandwidth* Expressed in that way, v Békésy's measurements shows an almost constant relative bandwidth (in the frequency range investigated which was up to 2000 Hz) of between 0 5 and 0 6 while Johnstone and Boyle's values for the cat was 0 22 at a frequency of 19 kHz Although this cat value is much smaller than those of v Békésy it is about three times higher than the relative bandwidth of the neural response areas (tuning curves) of single units in the rat cochlear nucleus which have values which vary from about 0 13 at 2 kHz to about 0 05 at 30 kHz

In this context it should be pointed out that the characteristics of the single units in the cochlear nucleus are not determined solely by the bandwidth of the tuning curves The high frequency part of the neural response curves in addition are extremely steep (cf Møller 1969) a fact which also may be important in frequency discrimination

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exhibit such prominent oscillations at high frequencies as it does at low frequencies when excited by a brief click.

Another method to estimate the system impulse response of the basilar membrane on the basis of unit responses in the eighth nerve was introduced by de Boer (1967). He computed the cross-correlation between the unit responses and a broad band noise used as stimulus and assumed this estimate to be a good approximation of the system impulse response of the basilar membrane. Furthermore, the outcome of such a calculation was assumed to mainly reflect the linear properties of the system under test and thus omits the nonlinearity of the neural excitatory process (de Boer and Kuyper 1968). The results presented by de Boer indicate that the system impulse response of the auditory frequency analyzer at the level of single units in the eighth nerve is much less damped than implied by the mechanical properties of the basilar membrane. Unfortunately, the method used by de Boer also suffers the same limitations as that used by Kiang and its use is thus restricted to units of low CF.

The experiments reported on in the present paper were designed to provide an estimate of the properties of the damped oscillatory response of the peripheral auditory spectrum analyzer on the basis of the firing rate of single units in the cochlear nucleus of the rat. The stimulus was arranged so that information about the oscillatory response of the basilar membrane could be obtained without influence of the time integration which occurs between the place of excitation of the receptor cells and the site of recording (cochlear nucleus). Paired short clicks, in which the interval between the two clicks could be varied satisfy this requirement. The response of a linear system to paired click excitation will be the sum of the response to the individual clicks. If the system has an oscillatory response to a short click, and the second click is applied before the response to the first click has died out, the *net* magnitude of the sum of the two responses will depend upon both the phase relation between the response to the first and the second click and on the amplitude of the response to the first click at the time the second click is applied. If the oscillatory response to the second click appears in opposite phase relative to that evoked by the first click the response to the second click will tend to cancel the response of the first click. The mean response to paired clicks will thus fluctuate as a function of the interval between the two clicks as long as the interval between the clicks is smaller than the duration of the oscillatory response of the system. Consequently the discharge rate of single neurons in the cochlear nucleus will show fluctuations as a function of the time separation between the two clicks. A histogram which shows spike number as a function of time between the clicks will thus show dips which occur with the same periodicity as the damped oscillation. When the interval between the two clicks is increased the magnitude of the dips will decrease at a rate which is in accordance with the decay of the oscillatory response of the system. Since the mean value of the neural firing rate of units in the cochlear nucleus can be assumed to represent the magnitude of the neural excitation it is possible to use these values to obtain a quantitative measure of the system impulse response of the peripheral auditory frequency

In the present investigation this method is used to study *to what extent* the frequency selectivity in single neurons in the rat's cochlear nucleus is accompanied by a concommitant oscillatory response to short click sounds, particular in those units with high CF The auto correlation of the impulse response is also computed from the responses to click pairs and compared with that computed from the unit's tuning curve

Methods

The recordings were made from the rat cochlear nucleus as described previously (Møller 1969 a) The clicks were generated by applying short rectangular impulses (10 μsec long) to a condenser microphone which was used as a sound source The pulses were generated by a Grass S4 stimulator in the first part of the study and the stimuli presented in bursts of 50 msec duration at a rate of 6/sec *The individual click pairs were presented at 5 or 10 msec* inte- [illegible] value
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cou [illegible] of the
stir [illegible] auto-
[illegible] generator

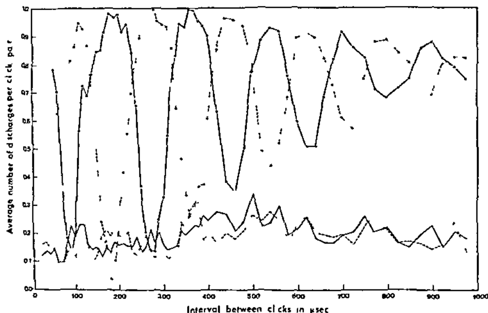
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recorded on the other track In later data processing these sync pulses started the sweep of an Intertechnique DIDAC 800 physioscope used *in its* post stimulus time histogram mode Short impulses (generated by the level discriminator to which the nerve discharges were fed) were fed into the input of the DIDAC Usually an analysis time of 200 msec/channel was used [illegible] each synchronizing pulse reset the address [illegible] the sweep In that way the sweep always [illegible] on the DIDAC could thus be set inde- [illegible] experimenter to choose the optimal time *resolution of the analysis Computation of the auto-correlation of the system impulse response* to click pairs and to pure tones was *performed* on an IBM 1800 digital computer and the results were plotted by a Calcomp plotter connected to the computer

Results

Fig 1 shows the average number of discharges from a unit (CF of 5.4 kHz) in response to paired clicks as a function of the interval between the clicks The response to clicks of equal polarity (rarefaction click *followed by rarefaction click*) is indicated by dots connected by solid lines and the responses to clicks *with* opposite polarity (rarefaction followed by condensation clicks) with dots connected by dashed lines The interval between the clicks was varied *between 50 and 1000 μsec* and the click pairs were presented in 50 msec long bursts with 10 click pairs *in* each burst The values *in* Fig 1 *show the mean response* per click pair in 32 *bursts* of clicks presented at a rate *of* 6 per second *It is seen that the responses* expressed in that way show minima at regular intervals Clicks with the same polarity produce minima at click intervals where clicks of opposite polarity *produce maxima* The amplitude of the oscillations *of the* average *response* decreases when the interval between the two clicks *is increased*

Fig 1 also shows the average firing rate during the first 50 msec following the end of the stimulus burst counted in the same way as the activity *during stimulation* (*cf* Møller 1969 a) Solid lines correspond to clicks of equal polarity and *dashed*



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clicks of equal and opposite polarity respectively

fines to clicks of opposite polarity It is seen that the first minimum (for stimulation with clicks of opposite polarity) represents a lower firing rate during stimulation than after, thus indicating depression of the unit's spontaneous activity

When clicks of opposite polarity are used, the second click will evoke an oscillatory response of opposite phase relative to that of the first click Consequently, the mean response will have minima when the click interval is equal to the time of an integral number of whole cycles of the damped oscillations Conversely, the response to clicks of equal polarity will show minima when the click interval is half one and a half two and a half etc times the duration of one cycle It is thus possible to determine the length of one single period of the damped oscillation of a system from its response to double clicks measured within a sufficient range of click intervals The results depicted in Fig 1 show that each period of the damped oscillation of this unit has a length of 180 μsec which corresponds to a frequency of 5 45 kHz The CF of the unit as determined from its tuning curve was 5 4 kHz The frequency of the damped oscillation is thus almost equal to the CF of the unit When the delay between clicks is increased the amplitude of the periodic fluctuations in the res nse

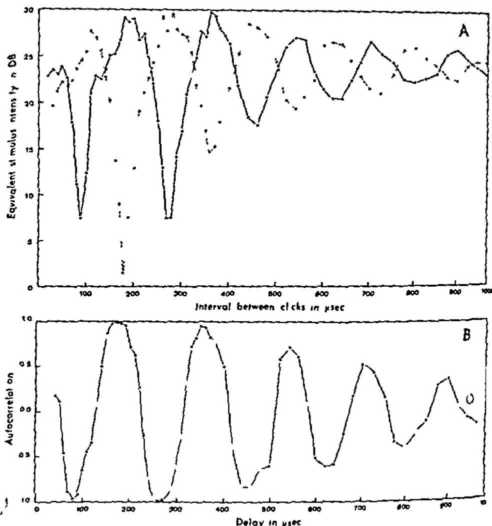


Fig 2 A The data shown in Fig 1 with the response converted to show equivalent stimul intensity in db as a function of interval between the individual clicks
B Auto-correlation of the system impulse response computed from the data shown in A

decreases indicating the damped character of the system impulse response Result as those shown in Fig 1 thus provide a quantitative measure of the rate of decay o the damped oscillation produced by a click

Although the system impulse response of a frequency selective system cannot be determined by this method the auto correlation of its impulse response can be deter mined The auto correlation is a function of the delay and it is the mean product of the response itself and its delayed version (The mathematical operations necessary for that are described in the Appendix) In order to ascertain these values the raw data expressed in Fig 1 have to be converted to show the change in sensitivity of the

unit (expressed in equivalent sound intensity instead of neural firing rate) This transformation was accomplished with the aid of a stimulus response relationship which shows firing rate as a function of sound level with the same type of sound used as stimulus (*cf* Moller 1969 a) Figure 2 A shows the data from Fig 1 converted in that way The ordinate shows equivalent sound level in dB instead of spikes per click sound Using these data the auto-correlation of the system impulse response was calculated The results are shown in Fig 2 B The decay of the oscillations in the auto-correlation of the system impulse response of a linear system is a direct measure of the spectral bandwidth of the system The impulse response of a system with a narrow bandwidth has a long duration and its auto correlation correspondingly shows a slow decay whereas a system with a large bandwidth has a brief impulse response the auto-correlation of which accordingly shows a rapid decay In order to compare the frequency selective properties of the auditory system disclosed by the above described experiments to those based on measurements with pure tones the auto-correlation of the system impulse response was also calculated on the basis of the unit s response to pure tones (see Appendix) In unit 68 1 auto-correlation was calculated both from iso-rate functions (curves which relate that given sound intensity which produces a certain firing rate as a function of tone frequency *cf* Moller 1969 a) and from tuning curves Fig 3 A compares the auto-correlation computed from the response to pure tones (solid line) and that computed from the response to paired clicks (dashed line and filled circles) It is seen that the data based on iso rate curves have a faster decay than those computed on the basis of the responses to click pairs The calculations were based on an iso-rate curve (Fig 3 D solid line) representing the sound intensity required to produce an average number of discharges of 4 7 per 50 msec toneburst (see Moller 1969 a) Similar results were obtained when the unit s tuning curve was used as a basis of this calculation. These results thus indicate that the auditory system shows a greater spectral resolution (smaller bandwidth) when stimulated with click sounds than with single pure tones It should be noted that the tuning curves and iso rate functions are based on the response to single pure tones while the click data represent the properties of broad band stimulation Both the iso-rate curves and the data from stimulation with click pairs were obtained at about the same sound intensity which was well above the unit s threshold Difference in sound intensity is therefore not likely to be responsible for the measured difference in spectral resolution with regards to clicks and tones

In order to estimate the magnitude of the difference in spectral resolution to clicks and tones the data computed from the response to double clicks were matched to the auto-correlation function based on a narrower response area The geometry of the new response area was similar to the unit s iso-rate function It was then found that the bandwith to paired clicks was about half of that of the unit s iso rate function. Fig 3 B shows in a similar way as Fig 3 A the auto correlation of the system impulse response based on the response to click pairs and that computed from an iso-rate curve which was half as wide as the measured curve but

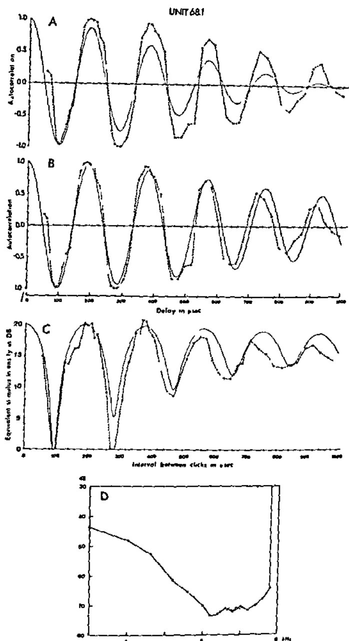


Fig 3 A Auto-correlation (dashed line) of the system impulse response computed from the response to paired clicks (see Fig 2 B) together with that computed from the units iso-rate function which is shown in D (solid line)

B Same as A but the solid line is the auto-correlation computed on the basis of a response area which is half as wide as the units iso-rate function (shown by dashed line in D)

C Expected response to paired clicks (solid line) of a model whose iso-rate function is half [illegible]

hed l[illegible]

shows the function the inverse Fourier transform of which is seen in D

of the same shape (dashed line in Fig 3 D) This method is of course, rather unrefined since we do not know whether the shape of the selectivity curves changes in ways other than becoming narrower, and also because of the uncertainty of the unit data The data shown in Fig 3 B, however, are seen to fit rather well except for a small deviation between the frequency of the oscillations

Correspondingly, a computation of the expected response to paired clicks of a model with a power transfer function of the same shape but half as wide as the tuning curve of unit 68 1 agrees well with the experimentally obtained unit response to paired clicks as can be seen in Fig 3 B and C The width and shape of the response area of the unit (the responses of which are shown in Fig 1—3) agrees well with the mean value of the width of other units with CF in the same frequency range (Møller 1969 a c)

The preceding results were further verified through the examination of five additional units (CF from 1 8 to 6 kHz) using the previously described method The results were in general agreement with those shown in Fig 1—3 Twenty five more units were studied by using an automatic method for varying the interval between the clicks The interval between the pulses was varied linearly at a rate of 0 1 Hz and the distribution of discharges as a function of the interval between two adjacent clicks was determined by an average computer used in its post stimulus time histogram mode The rapidity of this method made it possible to study the response under several stimulus intensities Fig 4 A—C shows a typical example of results from such an experiment The recordings were made from a unit in which the CF was near 20 kHz Each graph represents 100 seconds of data The clicks had the same polarity and the pairs were presented at a rate of 100 per second The delay between clicks was varied from 50 to 800 μsec and the intensity was varied in steps of 10 dB with 4 A representing the highest intensity It is seen that almost the same pattern is present at the three different click intensities shown The relative uncertainty in these data due to the short recording time makes quantitative determinations of the oscillatory properties less favorable than is the case for the data shown in Fig 1—3 A theoretical calculation of the expected response to paired clicks on the basis of the unit s tuning curve was made in the same way as that of the previously described unit This computation which is seen in Fig 4 D expresses general agreement with the experimental results obtained from stimulation with paired clicks On the other hand the fluctuations as a function of click interval can be seen to persist longer in the experimental data (A—C) than in the curve showing expected response (D) similar to what was seen in Fig 3 These results thus indicate that the unit has a smaller bandwidth when stimulated with broad band sounds than it has when the stimuli were single pure tones The tuning curve of the unit (Fig 4 E) belongs to the common type (*cf* Møller 1969 a)

The responses to paired clicks of most of the 25 units investigated with CF ranging from 1 8 to 30 kHz had a similar characteristic as those shown in Fig 4 A—C although the magnitude of the oscillations varied somewhat among different units Fig 5 A—D depicts responses from two units which represent the extremes

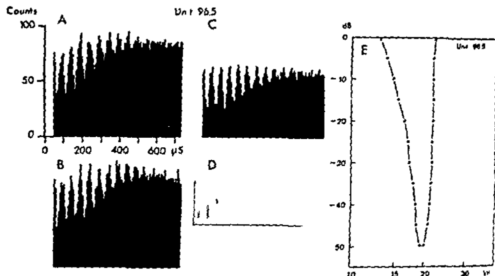


Fig 4 A—C are histograms of the response to paired clicks (of equal polarity) showing spil counts as a function of interval between the clicks Sound intensity was —10, —20, —30 d respectively

D Expected response calculated on the basis of the unit s tuning curve shown in E

E The unit s tuning curve

of the variation The CF of the two units had nearly the same value (for unit 81 6 i was 26 5 kHz and 30 5 kHz for unit 82 11) and the units tuning curves are seen i Fig 5 E Three min of activity were processed and the stimulus click pairs were presented with a repetition rate of 100 pps in both cases Graphs A and C illustrate responses to clicks of opposite polarity while B and D represents the response to clicks of equal polarity Graphs A and B are from a unit with unusually large oscillations while C and D represents a unit with very small oscillations It is thus clear that even units with a high CF near the upper limit of response (about 30 kHz) can produce a clear oscillatory response to click stimulation

Most of the units from which recordings were made had tuning curves with a shape similar to those units whose response has been previously described A few units however deviated from that common pattern and some of these had a tuning curve with two peaks instead of one indicating a double resonance The response of such a double peaked unit to stimulation with paired clicks is shown in Fig 6 The tuning curve for this unit is seen in Fig 6 G

Contrary to what was found for the units with single peaked tuning curves the double peaked unit showed a pattern of oscillations which changed with the sound intensity Near threshold (−40 −30 dB D E) the curves show oscillations at a frequency which agrees with that of the highest best frequency (12 kHz) At −20 and −10 dB the graphs (A–B) suggest a strong component whose frequency coincides with the lowest best frequency 4 kHz At the highest intensity used (−10

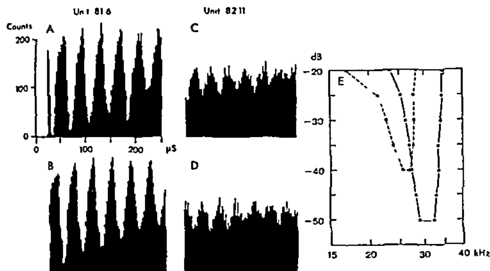


Fig 5 A—D histograms of response to paired clicks of two units, one with a CF of 26 5 kHz (A—B) and one with a CF of 30 5 kHz (C—D) A and C represent clicks of opposite polarity, B and D clicks of equal polarity
E shows the tuning curves of the units (unit 81 6 by dashed line and unit 82 11 by solid line)

dB, A) the oscillations which correspond to the highest best frequency nearly disappeared

Fig 6 C shows the expected response to double click stimulation on the basis of the response areas to pure tones (tuning curves) This curve is similar to that depicting the responses elicited by paired clicks of low intensity

In a linear system with multiple resonances the system impulse response will show a mixture of damped oscillations each one corresponding to a particular resonance The relationship between the amplitude of these components corresponds to the amplitude of the resonances but this relationship is independent of the intensity of the excitation The shape of the resulting net responses of a linear system is thus independent of the intensity of the excitation Results shown in Fig 6 indicate that the summation of the oscillatory components corresponding to the two resonances of the double peaked unit depends on the stimulus intensity The high frequency resonance dominates at low intensities while the low frequency resonance dominates at high intensities

In stimulation with paired clicks the shape of the response pattern changes with sound intensity thereby indicating a nonlinear action in the summation of the contribution to the response from the high frequency and the low frequency areas

Discussion

The results of the present investigation show that the peripheral auditory frequency analyzer, up to the level of the neural excitation responds to a transient sound with

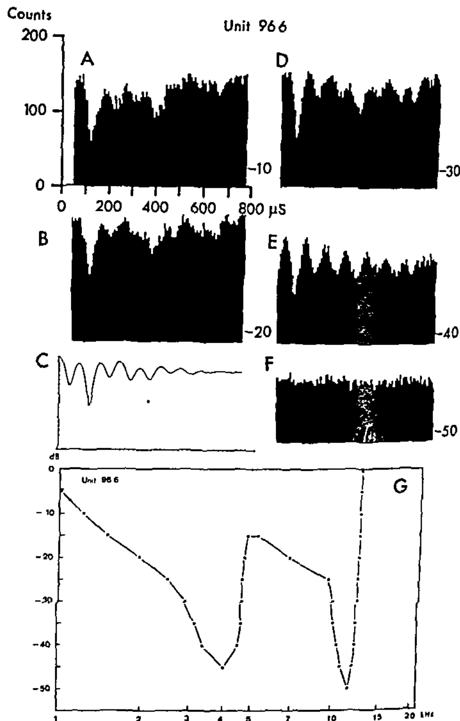


Fig 6 Response of a unit with a two-peaked tuning curve
A, B and D—F are histograms of response to paired clicks equal polarity Legend numbers indicate sound intensity
C Expected response calculated on the basis of the unit's tuning curve shown in G
G The tuning curve of the unit

a damped oscillation whose frequency is equal to the unit's CF The auto correlation of the system impulse response has been determined on the basis of the responses of single units in the cochlear nucleus to stimulation with double clicks These results show that units which CF_s cover a large frequency range exhibit a damped oscillation in response to transient sounds It was also found that this oscillatory response persisted in a large intensity range with about the same rate of decay The rate of decay of the auto correlation of the system impulse response (computed from the responses to paired clicks) was slower than that of the auto correlation computed from the response to single pure tones (iso-rate functions or tuning curves) These results thus agree well with those obtained in experiments using broad band noise (Møller 1969 c) which showed that the bandwidth of single units in the cochlear nucleus was smaller in response to broad band noise than the width of the tuning curves

The selectivity of single units in the auditory nerve and the cochlear nucleus (Kiang 1965, Kiang 1968 and Møller 1969 a c) is much greater than implied by measurements of the vibratory pattern of the basilar membrane (v Békésy 1960 and Johnstone and Boyle 1967) It is generally assumed that the sharpening of the neural responses when these are compared to the response of the basilar membrane occurs somewhere in the nervous system The results of several investigations indicate that there exists a mechanism similar to that known as lateral inhibition (quantitatively studied in the visual system by Hartline see e g Hartline 1967) which in the auditory system enhances the spectral contrast by sharpening the response areas as they appear when mapped by single pure tones The sharpening of the tuning curves relative to the mechanical resonance curves of the basilar membrane could very well have its origin in an interaction between the rectified and time integrated output from a number of hair cells located in a narrow region of the basilar membrane

The results of the present study support the existence of a sharpening mechanism but additionally the results furthermore indicate that the sharpening of the neural selectivity curves is *not* accomplished by interaction between the rectified and time integrated output from a number of elements placed along the basilar membrane If lateral inhibition is based on manipulations of the time integrated output of the hair cells such small bandwidth values could not have been measured with the method of the present investigation since this method only reflects the *oscillatory* response of the system under test

The spectral selectivity of the peripheral auditory frequency analyzer is thus either the result of an interaction between the activity in the hair cells *prior* to a level where any appreciable time integration of the excitation occurs or it is a result of the mechanical properties of the basilar membrane itself The selectivity shown in the present study can however not be the result only of those mechanical properties of the basilar membrane which were studied by v Békésy (1960) and Johnstone and Boyle (1967) since the basilar membrane according to them has resonance curves which are 5—10 times wider than the bandwidth values obtained in the present study

In this context it should be mentioned that Kiang (1965) has shown that the response to a tone by single primary fibers in the eighth nerve can be inhibited by another tone even though the central connection of the nerve is interrupted. Furthermore, recent studies (Kiang 1968) have shown that this inhibition is present after degeneration of the efferent terminals on the hair cells. Degeneration was due to sectioning the crossed and uncrossed olivo-cochlear bundle.

The fact that the response to paired click sounds implies bandwidth values which are smaller than those of the tuning curves is in agreement with results obtained using broad band noise (Møller 1969 c). The present study thus supports the results obtained using broad band noise and showing that the resolution of the auditory spectral analyzer is larger for sound with a broad spectral distribution (noise and paired clicks) than it is for single pure tones (tuning curves and iso-rate curves).

It has been suggested earlier that a purely neural frequency selectivity is responsible for the frequency discrimination of the auditory system. If this should be the case, the response of the units to paired clicks would be independent of whether clicks of equal or opposite polarity were used.

The units examined in the present investigation responded with a sustained train of discharges to stimulation with continuous tones within their response areas (cf. Møller 1969 a). All units had a different response pattern to stimulation with clicks of equal polarity and with clicks of opposite polarity.

The results thus indicate that the frequency analysis represented by the tuning curves of single units in the cochlear nucleus is not based on a time interval analysis. If this had been the case, the response would have been the same irregardless of whether clicks of opposite polarity or clicks of the same polarity were used. This is obviously not the case as is evident from Fig. 1 and 5 of the present paper.

There are, however, some units in the cochlear nucleus which only responded to transient sounds (cf. transient units, Møller 1969 b). Those units had been shown to have a selectivity with respect to repetition rate or rather the duration of silence between adjacent sounds. The response of such units, however, was independent of whether the stimulation consisted of clicks of equal or opposite polarity. It was therefore concluded that the response of these latter units was not oscillatory.

Appendix

The determination of the autocorrelation of the impulse response of a linear system from the mean value of its response to excitation with double impulses is based on the following.

The output of a linear system is the convolution between the system impulse response and the input. Correspondingly it can be shown that the autocorrelation of the output of a system is the convolution between the autocorrelation of the system impulse response and that of the input (see e.g. Lee 1960).

Eq. 1

$$C_{oo}(\tau) = \int C_{ss}(t) \; C_{ii}(\tau - t)\, dt$$

where $C_{oo}(\tau)$ is the autocorrelation of the output as a function of the delay of a system which is specified by the autocorrelation $C_{ss}(t)$ of its impulse response to an input the autocorrelation of which is $C_{ii}(t)$. Furthermore the autocorrelation at zero delay $C_{oo}(0)$ is equal to the mean power of the output. Thus we can determine experimentally (cf. Fig. . .) Eq. 1 that reduces to only include $C_{oo}(0)$

Eq. 2

$$C_{oo}(0) = \int C_{ss}(t) \; C_{ii}(t)\, dt$$

Correspondently, the convolution between the auto correlation of the stimulus and that of the system impulse response becomes simple and has only 3 discrete values of which two are equal

$$C_{oo}(\tau) = \int C_{hh}(t) \quad C_{ii}(\tau - t)dt$$

$$\begin{aligned} C_{ii} &= 2k \quad \text{for} \quad t = 0 \\ &= k \quad \text{for} \quad t = \Delta t \\ &= k \quad \text{for} \quad t = -\Delta t \end{aligned} \qquad \text{Eq 3}$$

where k is the amplitude of the individual clicks and Δt is the interval between the two clicks As mentioned above the auto-correlation of the response at zero delay is a measure of the total power We thus only need to carry out the calculation for $\tau = 0$ The output as a function of Δt is then

$$C_{oo}(0, \Delta t) = 2k \quad C_{hh}(0) + k \quad C_{hh}(\Delta t) + k \quad C_{hh}(-\Delta t) \qquad \text{Eq 4}$$

Since the auto-correlation function is an even function, the auto correlation of the system impulse response $C_{hh}(\Delta t)$ at negative delays is equal to that at positive delays The output is thus

$$C_{oo}(0, \Delta t) = 2k(C_{hh}(0) + C_{hh}(\Delta t)) \qquad \text{Eq 5}$$

For simplicity we denote the output C_{oo} $(0, \Delta t)$ by $R^2(\Delta t)$ The unit response to paired clicks gives R^2 as a function of Δt and we seek the auto-correlation C_{hh} of the system impulse response We first normalize $C_{hh}(\Delta t)$ by dividing all its values with $C_{hh}(0)$

With the average response to clicks of equal polarity named R_+^2 (Δt) we obtain

$$R_+^2(\Delta t) = 2k(1 + C_{hh}(\Delta t))$$

$$C_{hh}(\Delta t) = \frac{R_+^2 (\Delta t)}{2k} - 1 \qquad \text{Eq 6}$$

where $C_{hb}(\Delta t)$ is the normalized auto-correlation Since the factor k is unknown we need another equation in order to find $C_{hh}(\Delta t)$ That equation is obtained from the response to clicks of opposite polarity The auto-correlation $C_{ii}(\Delta t)$ of that sound is in accordance with Eq 3

$$\begin{aligned} &2 \text{ k for } t = 0 \\ &- \text{k for } t = \Delta t \\ &- \text{k for } t = -\Delta t \end{aligned}$$

Thus in the same way as Eq 6 is obtained

$$R_-^2 (\Delta t) = 2k(1 - C_{hh}(\Delta t))$$

$$C_{hh}(\Delta t) = 1 - \frac{R_-^2 (\Delta t)}{2k} \qquad \text{Eq 7}$$

We look for the ratio between $R_-^2(\Delta t)$ and $R_+^2(\Delta t)$ Since we assume that the auto-correlation of the impulse response to a negative impulse is the same as that to a positive one

$$\frac{R_+^2 (\Delta t)}{2k} - 1 = 1 - \frac{R_-^2 (\Delta t)}{2k} \qquad \text{Eq 8}$$

We can thus determine k from Eq 8

$$\frac{1}{2k} (R_-^2 (\Delta t) + R_+^2 (\Delta t)) - 2 = 0$$

$$k = (R_-^2 (\Delta t) + R^2 (\Delta t))/4 \qquad \text{Eq 9}$$

With k known $C_{hh}(\Delta t)$ can be determined from either (6) or (7)

The auto-correlation of the system impulse response was also computed on the basis of the units' tuning curves The tuning curves were assumed to represent the power transfer function of a linear system The computations were based on the fact that a linear system's power transfer function and the auto-correlation function of its impulse response are Fourier transforms of each other Thus

$$C_{hh}(\tau) = \int_{-\infty}^{+\infty} F(\omega)\cos(\omega\tau)d\omega \qquad \text{Eq. 10}$$

where $C_{hh}(\tau)$ is the auto-correlation of the system's impulse response as a function of the delay τ, $F(\omega)$ is the power transfer function of the system as a function of ω ($\omega = 2\pi f$ where f is the frequency). (Usually the normalized auto-correlation is used which has a value of 1 at zero delay.)

The expected relative response $R^2(\Delta t)$ to paired clicks of a system with transfer function $F(\omega)$ was furthermore determined on the basis of the computed auto-correlation function $C_{hh}(\tau)$. According to Eq. 5 the average response to clicks of equal polarity is

$$R^2_{\pm}(\Delta t) = C_{hh}(0) \pm C_{hh}(\Delta t) \qquad \text{Eq. 11}$$

$R^2_{\pm}(\Delta t)$ is the relative response as a function of the interval Δt between the clicks. (The factor k is omitted for simplicity.)

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Slowly Adapting Muscle Receptors in Man

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Abstract

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Unitary nerve impulses originating from slowly adapting muscle receptors were recorded with percutaneously inserted tungsten electrodes from the median and the tibial nerves in awake human subjects Discharges from 72 receptors located in the wrist flexor muscles and the ankle extensor muscles were analysed The possibilities to derive criteria of muscle spindles and Golgi tendon organs were tested by means of a number of experimental procedures A differentiation between these two types of receptors could, for technical reasons, not be achieved by electrically induced muscle twitches For the majority of the endings muscle spindle characteristics could be demonstrated In relaxed muscles a number of the muscle spindle afferents exhibited a high dynamic sensitivity to passive joint movements whereas a smaller proportion of them had a low dynamic sensitivity A steady state discharge as a function of the muscle length could be demonstrated for some of them During weak voluntary contractions without *external muscle shortening, the majority of the units responded with a sustained* impulse discharge which started and stopped almost simultaneously with the onset and the cessation of the extrafusal contractions It was concluded that the motor cortex exerts a very powerful control of the intrafusal muscle fibres of the majority of the muscle spindles For a few units the relation between the muscle force and the impulse frequency was very striking but for the majority of them this relation was poor It was concluded that the former units were Golgi tendon organs

Impulse discharges in muscle nerves have earlier been studied in awake human subjects with the method introduced by Vallbo and Hagbarth (1967 1968) Multifibre recordings and a limited number of single unit recordings (Hagbarth and Vallbo 1968 b, Hagbarth and Vallbo 1969) suggest that the afferent activity from muscle spindles increases during voluntary contractions. This is consonant with the notion that the muscle spindles play a significant role in the control of this type of muscular activity However in these studies there was some difficulty to ascertain from which type of receptor a discharge originated

In the present investigation, it will be shown that a number of tests can be employed in order to differentiate between the various types of intramuscular receptors although electrically induced muscle twitches or conduction velocity measurements could not be used Some of the important tests involved voluntary contractions Further, a survey is given of the various types of unitary discharges encountered and

it will be shown that the majority of the endings exhibited sustained discharges during sustained voluntary contractions without external shortening

Methods

The subjects were 15 healthy adults, age between 20 and 35 years 72 afferent units were studied in 28 expts The tibial nerve was exploited in 8 of these experiments and the median nerve in the other 20 The study was confined to receptors located in the forearm muscles proximal to the wrist and receptors located in the leg muscles proximal to the ankle joint. Units with end organs located in the hand or in the foot were disregarded

Essentially the same technique as described before was used (Vallbo and Hagbarth 1968 Hagbarth and Vallbo 1968 a, 1968 b, 1969), but minor modifications have been undertaken continuously in this series of experiments A brief account will be given of the latest version of experimental procedures, whereas earlier modifications will be described only as much as they may be relevant for the interpretation of the findings The tibial nerve was searched 10—20 cm proximal to the knee joint the median nerve 10—15 cm proximal to the elbow As a preliminary a stimulating needle electrode was inserted towards the nerve and single pulses were given from a battery driven stimulator When a contraction was obtained in the appropriate muscles in response to the minimal single shock a recording electrode was inserted parallel to the stimulating electrode and to approximately the same depth If no sign of nerve activity was noticed the electrode was withdrawn for some distance, but not through the skin and it was reinserted with a slightly different angle This was repeated until the electrode tip was in a bundle of fibres distributed to the deep structures of the leg or the forearm as indicated by several signs (Vallbo and Hagbarth 1968, Hagbarth and Vallbo 1968 a, 1968 b 1969) Once the electrode tip was in the right nerve bundle the electrode position was adjusted in very small steps, and the activity was carefully examined after each adjustment The majority of the single units were found when taps and sustained pressure were applied on the muscle bellies or muscle tendons Passive joint movements and voluntary contractions were also employed but these tests seldom revealed a unit which later could be identifeid as afferent Only small passive joint movements and weak voluntary contractions were tried in order to avoid displacement of the recording electrode For instance, in the sequences illustrated in Fig 6 and 7 the torques due to voluntary contractions of the flexor muscles acting on the index were 0 1—0 2 meter newton The maximal torques which can be produced in the same types of contraction were tested in three subjects It was found that the mean value was 4 1 meter newton *i e* 20—40 times larger

The recording electrodes were 0 2 mm diameter tungsten needles pointed electrolytically to a tip diameter of 5—15 μ and insulated as described in detail before (Hubel 1957, Vallbo and Hagbarth 1968) After the coating the laquer covering the tip of the electrodes was removed by inserting the electrode through a piece of cloth several times until the electrode impedance had diminished to 75—150 kohm, as measured at 1000 Hz The uncoated tip area was estimated under microscope for a number of electrodes and related to their impedances. [illegible] of 100 kohm was insulated [illegible] 0 μ^2 [illegible] in 50 [illegible] was [illegible] e fre- [illegible] gure [illegible] trode needles inserted in the vicinity of the intraneural electrode served as the [illegible] and the ground connection These two electrodes were coated to within 3—5 mm from the tip The recording electrodes were all through the experiment manipulated by the experimenter with a pair of tongs the branches of which were covered with rubber tubings which had the function to protect the electrode coating and to give a firm grip of the electrode shaft

The subject was lying on a bench with his face downwards In the experiments on the median nerve his upper arm extended laterally over the border of the bench his forearm was directed towards the foot end of the bench and it rested on a shelf which was tilted by 30 towards the floor The volar aspect of the hand and the forearm was *directed* upwards His hand was attached to a device for measurements of the wrist joint angle and the twisting force between the forearm and the hand Flexions and extensions at the wrist joint were allowed whereas all movements at the finger joints were impeded The subject's hand was fixed by means of ribbons to a plexiglass plate which could rotate around an axis which coincided with the main axis for flexions and extensions at the wrist A potentiometer supplied by a DC-potential, was connected to the axis of the hand plate and the angle at the wrist was recorded as the output potential of this potentiometer The relation between the recorded potential and the plate angle was linear and accurate within ±0 5 degree Control of the wrist

joint angle and measurements of the torque were achieved by means of a mechanical device and a strain gauge system connected to the hand plate A heavy steel band extended radially from the axis of the hand plate To the outer end of this band a steel rod was fixed parallel to the plate axis The other end of this rod was rigidly attached to one face of an aluminium wheel close to its periphery This wheel could rotate around an axis which coincided with the extension of the hand plate axis Therefore, the hand the hand plate and the wheel formed a system which rotated as a block around the same axis Further the wheel could be fixed in any position and the neural activity could be studied while muscle shortening during voluntary contractions was restricted to a minimum The changes of the torque between the hand and the forearm of the subject was recorded as the output of a strain gauge system. This consisted of a carrier amplifier (Sanborn Model 311 A) and two strain gauges glued on the steel band which formed the mechanical link between the hand plate and the wheel as described above This system involved an error in the steady state as the measured torque was the sum of the twisting forces between the hand and the forearm on the one hand and the twisting force due to gravity force acting on the hand plate on the other This last component varied with the angle but this variation was small and amounted to a maximum of approximately 0 05 mN The output signal from the strain gauge system was calibrated in meter newton, abbreviated mN

The subject's hand was fixed in a position which was the same from one experiment to the other and the basic observations were done at this initial position In most of the experiments (16 expts) the wrist joint angle was 180°, the angles at the finger base joints were 140° and the angles at the interphalangeal joints were 180° In four experiments the angles were 180 at the wrist the finger base joints and the interphalangeal joints In eight early experiments the subject was sitting instead of lying and his forearm rested on its medial side on a table This position implied the disadvantage that large parts of the wrist flexor muscles were not accessible for local mechanical stimuli

A similar system for measurements of the joint angle and the torque as the one described for the hand was used in the experiments on the leg

The electromyograph in record was obtained with surface electrodes consisting of short tube of plexiglass 10 0 mm in diameter and 3 0 mm in height. A silver plate was glued on one of the flat surfaces forming a lid over the end of the tube The connection to the amplifier was soldered to this plate The hollow of the tube was filled with electrode jelly (Cambridge Instrument Co Ltd London) and the electrode was attached to the skin over the muscles by means of a ring of double sticking tape on the flat surface opposite to the silver plate (Stomaseal, Minnesota Mining & Manufacturing Co) Two of these electrodes were connected to one half of a Tektronix plug in unit 3A3 in a Tektronix oscilloscope 565

Local pressures were applied over the muscle bellies and tendons by means of a strain gauge [illegible] ne type as [illegible] t with the [illegible] ommercial [illegible] A probe [illegible] de of the vibra [illegible] amplitude was [illegible] unloaded The [illegible] force was ±6 0 newtons and independent of the frequency It might be added that the stimulus from the vibrator was just strong enough to induce a weak tonic vibration reflex (*e g* Eklund and Hagbarth 1966 deGail Lance and Neilson 1966) in the flexor muscles of one single finger in most of the subjects tested when the stimulus was applied at the level of the base joint

An 8-channel tape recorder Precision type 6208) was used for recording the following events during the experiments 1 the nerve activity 2 the electromyographs of the appropriate muscle group (3) an analogue signal of the wrist or ankle joint angle as the case may be (4) an analogue signal of the torque at the wrist or at the ankle joint 5 an analogue signal of the force of the local mechanical stimuli (6 a time and marker signal and 7 comments and protocol on a voice channel The tapes were afterwards played back and the signals were photographed with a Grass camera from an oscilloscope An instantaneous frequency meter of the type described by Green 1967) was utilized for the analysis of some experiments (Fig 3 6 7 8

Results

The present report is based upon recordings from 72 afferent units with slowly adapting receptors located in deep structures Another 30 units were encountered

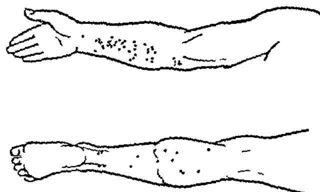


Fig 1 Location of the receptors Each dot indicates the point at which the threshold of a single unit was minimal for local mechanical stimuli

which probably belonged to the same group but they were excluded as they did not fullfil all the requirements of afferent discharge from deep structures The majority of the units (57 units or 79 %) were recorded from the median nerve and some (15 units or 21 %) from the tibial nerve All the units could be activated by local mechanical stimuli In Fig 1 are indicated the points of maximal sensitivity for such stimuli, which points were assumed to correspond to the locations of the receptors Five arm units are not indicated as the protocol was not clear with the regard to the location of these endings It is seen that the receptors were located on the posterior side of the leg proximal to the ankle joint and on the flexor side of the forearm, proximal to the wrist It seems that the receptors in the arm were evenly distributed within the area in which the muscles innervated by the median nerve are located However there is probably an over-representation of distally located receptors as the proximal parts of the wrist flexor muscles were not accessible for mechanical stimulations in eight experiments (see p 31) Twenty five percent of the arm units were collected in these experiments It might be claimed that nine of the endings could be joint receptors as they were located close to the elbow (7 units) the wrist (1 unit) or the knee joint (1 unit) However eight of them were activated by small voluntary contractions without changes of the joint positions and five of them were activated by small passive movements of the joint distal to the one at which the receptors were located These findings are not consonant with the notion that the endings were joint receptors

For each unit a point or a small area could be defined in which the sensitivity to mechanical stimuli was maximal and there was as a rule a larger continuous field of lower sensitivity around this area Some of the afferents in the arm could be activated not only by stimuli applied to a point over the muscles bellies but also by stimuli applied to the tendons 2 3 cm proximal to the wrist (Fig 2 B right) but apart from such cases the receptive field for local stimuli was continuous for any one unit The possibility that the units were efferents which were reflexly activated by the local stimuli was considered However the response to the mechanical stimuli were very distinct and the impulse activity was very closely related to the stimulus

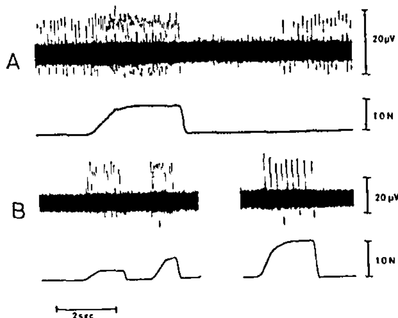


Fig 2 Single unit responses to local pressures Upper traces show nerve impulses lower traces analogue signals of the local pressures A A continuously discharging receptor in the arm located 6 cm distally to the medial epicondylus of the humerus The pressure was applied at the point of minimal threshold B A receptor in the arm located 12 cm distally to the medial epicondylus of the humerus The left hand part shows responses to pressures at the point of minimal threshold and the right hand part shows the response to a pressure on the flexor muscle tendons at the wrist i e approximately 17 cm distally to the location of the ending Upward deflections indicate positive signals at the intraneural electrode in this and all other figures The force is given in newton (N)

intensity and time course (Fig 2) After discharges were never seen and on repeated, similar stimuli the same impulse activity appeared If it was at all a possibility that the local stimuli induced efferent discharges in α motor fibres which could be mis taken for afferent activities then an EMG activity would be seen at least occasional ly with a time course and an intensity closely related to the stimulus Such an EMG activity was never seen Any unit encountered which did not fulfil these requirements with regard to the location and the continuity of the receptive field and the discharge characteristics on local stimuli was disregarded in order to eliminate the pos sibility that remote skin receptors or reflexly activated efferent units would be in cluded

The units were all slowly adapting For 86 % of them this could be demonstrated by local pressures which induced a sustained impulse activity (Fig 2) Some of the units were already continuously discharging in the absence of local stimuli (see p 6) In these cases the impulse frequency increased on local pressures and as a rule there was a marked pause after the stimulus (Fig 2 A) The remaining 14 % of the units responded only during the rising phase of a local pressure but they

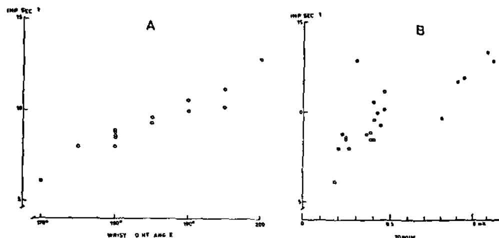


Fig 4 Relation between the wrist joint angle and the impulse frequency (A) and relation between the twisting force at the wrist joint and the impulse frequency (B) of a single unit in the arm The open circles indicate the impulse frequencies when the muscles were relaxed as measured 15 sec after passive changes of the wrist joint angle The filled circles indicate the impulse frequencies when the subject made sustained contractions against a fixed resistance of his flexor muscles acting on his third digit The same unit as in Fig 3 A and Fig 5 B The torque is given in meter newton (mN)

chosen zero level (Fig 4 B) The open circles represent the data obtained at various angles at the wrist when the muscles were relaxed and the filled circles represent the data obtained during voluntary contractions of the flexor muscles acting on the third digit against a fixed resistance This contractions was the most effective one to induce a discharge in this unit The plot suggests that the impulse frequency was lower at corresponding twisting forces during active contractions than it was under passive conditions This finding militates against the notion that this unit was a Golgi tendon organ as it has been shown in animal experiments that these receptors are either equally *sensitive to active and passive forces* or they are more sensitive to active forces (Jansen and Rudjord 1964 Alnaes 1967 Houk and Henneman 1967) It is therefore likely that this activity originated from a muscle spindle and it seems that this type of analysis offers a possibility to differentiate between afferents from *muscle spindles and Golgi tendon organs*

The dynamic sensitivity to muscle stretch was estimated qualitatively *Two* examples of impulse discharges during joint movements are shown in Fig 5 *It is seen* that one of these two units (Fig 5 A) had a high dynamic sensitivity whereas the other one did not have any conspicuous dynamic sensitivity (Fig 5 B) Fig 5 A and 3 B were taken from the same unit and Fig 5 B and 3 A were both taken from another unit The slow change of the impulse frequency after the stretch as seen in Fig 3 A can probably be ascribed to yielding in viscous elements between the angle recording device and the receptor and this type of slow decrease of the discharge rate was not accepted as a criterion of high dynamic sensitivity Of the 35 units which responded to passive joint movements 66 % had a definite dynamic sensitivity

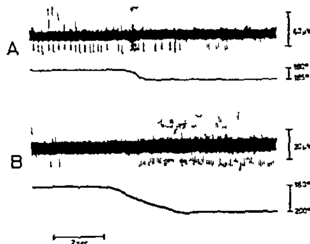


Fig 5 Responses of two units during passive changes of muscle lengths in relaxed muscles to indicate differences in dynamic sensitivities Upper traces show nerve impulses lower traces wrist joint angle A An arm receptor located 13 cm distally to the medial epicondylus of the humerus Same unit in Fig 3 B B An arm receptor located 6 cm distally to the medial epicondylus of the humerus Same unit in Fig 3 A and Fig 4

to this type of stimulus and 14 % had a very poor dynamic sensitivity similar to the one illustrated in Fig 5 B For the remaining 20 % sufficient information was not obtained on this point. Most of the units in this last group responded with only a few impulses during the movements It seems likely that the afferents which had a high dynamic sensitivity originated from muscle spindle primary endings Cooper 1959 1961 Harvey and Matthews 1961 Appelberg 1962 Bessou and Laporte 1962 Matthews 1963)

The findings concerning the two units illustrated in Fig 3 and 5 seem to justify the conclusion that one was a group II afferent and the other one was a group Ia afferent. The unit illustrated in Fig 3 A and 5 B exhibited a discharge which was closely related to the muscle length (Fig 4 A) but poorly related to the muscle force (Fig 4 B) It was therefore very likely a muscle spindle ending and not a Golgi tendon organ Further as it had a low dynamic sensitivity (Fig 5 B) it was probably group II afferent The unit illustrated in Fig 5 A and 3 B on the other hand had high dynamic sensitivity (Fig 5 A) and it exhibited indications of intrafusal con actions (see p 10) It was therefore very likely a group Ia afferent rather than a ıp Ib or a group II afferent How far the discharge characteristics shown in Fig and 5 are representative for the two types of muscle spindle afferents can not be lefined on the basis of the present material

It has been shown that muscle spindle primary endings in the cat are more sen tive to vibratory stimuli than secondaries and tendon organs in relaxed muscles Bianconi and van der Meulen 1963 Brown Engberg and Matthews 1967) In the investigation the response of the endings to vibration was studied for 21 units the arm The stimulus was applied at the point of maximal sensitivity It was and that seventy two percent of these endings could be driven i e they responded ith one or several impulses on each cycle of the stimulus The maximum frequency t which driving occurred varied between 50 Hz and 400 Hz Lower freque

than 50 Hz were not tried This range of maximal frequencies is similar to the range found by Bianconi and van der Meulen (1963) for muscle spindle primary endings in cat extensor muscles These investigators also found that all the group Ia afferents but only 45 % of the group II afferents could be driven by vibration. Further Brown *et al* (1967) have demonstrated that the tendon organs are largely insensitive to vibration in relaxed muscles and none of them could be driven at 100 Hz with their method of stimulation It seems therefore likely that the majority of the endings which followed the vibration frequency in the present investigation were spindle primary endings However, the sensitivity to mechanical vibration as applied over the receptor is probably a function of the depth at which the ending is located and not only of the properties of the receptor itself Attempts were also made to activate the endings by vibration on the muscle tendons at the wrist but these attempts were largely unsuccessful It was felt that the vibrator was not strong enough for this test

Lundberg and Winsbury (1960) have shown that fast slight pulls on the muscle tendons elicit discharges in the group Ia afferents with a lower threshold than in group Ib and group II afferents in relaxed muscles in the cat In the present study light taps on the tendon were tried in 18 units and it was found that 89 % of them responded mostly with one single impulse to such taps Occasionally two or three impulses were induced by one tap It seems justified to regard a discharge of this type as a support for the notion that the afferent was a group Ia fibre Most of the units which responded to tendon taps could also be driven by vibratory stimuli Of the 14 units which were tested with the two types of stimuli 12 responded to both of them One unit did not respond to any of the two stimuli and one unit responded to tendon taps but not to local vibrations

It has been shown in animal experiments that afferents from muscle spindles subject to fusimotor drive may exhibit an irregular discharge whereas the activity from Golgi tendon organs or deefferented spindles is regular (Eldred Granit and Merton 1953 Cooper and Daniel 1957 Crowe and Matthews 1964 Koeze Phillips and Sheridan 1968) In the present study pronounced differences with regard to the regularity of the impulse discharge rate were found between different endings Examples are given in Fig 3 which shows the instantaneous frequencies associated with passive joint movements for two different units The greater spreading of the points in B clearly demonstrates that the discharge was much more irregular for this unit than for the unit illustrated in A For eight afferents the impulse frequency was either markedly irregular or there were long term changes of the discharge frequency while the experimental situation remained unaltered These findings were interpreted as signs of intrafusal contractions which in turn indicated that the discharge originated from muscle spindles

The responses of 55 receptors were studied during voluntary contractions against a fixed resistance For as many as 87 % of them these contractions were associated with an increase of the discharge either during the whole period of contraction (67 %) or during a limited part of this period (20 %) The most common type in

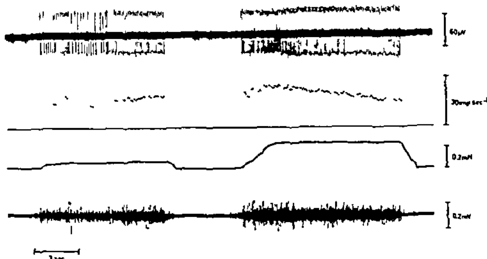


Fig 6 Afferent unitary discharge associated with two successive voluntary contractions without external shortening of the flexor muscles acting on the index From above are shown the unitary nerve impulses the instantaneous impulse frequency the torque due to the muscle contractions and the EMG activity recorded with surface electrodes over the flexor muscles 10 cm distally to the medial epicondylus of the humerus Straight line indicates zero impulse frequency The torque is given in meter newton (mN)

this last group were units which exhibited a short lasting discharge at the onset of the contraction More complex types of activities were occasionally seen such as an initial decrease of a continuous discharge followed by an increase of the impulse frequency as the intensity of the contraction increased For another 7 % of the endings the activity decreased during the voluntary contractions and 6 % did not respond at all There were some indications that the unitary discharges which were *non sustained* during voluntary contractions in many cases originated from muscles which were not prime executors of the particular type of contractions which was used as a test An example of a unit exhibiting a sustained discharge is given in Fig 6 From above are shown the nerve impulses the instantaneous impulse frequency, the measured changes of the torque at the wrist and the EMG activity recorded with surface electrodes over the flexor muscles when the subject made two successive contractions of the flexor muscles acting on his index It is seen that the impulse discharge started simultaneously with the increase of the force as recorded here, or approximately 0 5 sec after the first EMG activity and it stopped almost exactly at the moment when the force began to fall It was a typical finding for the majority of the endings which were sufficiently well studied in this respect that the unitary discharge was limited to the period during which other manifestations of the voluntary activation could be demonstrated Only exceptionally did the afferent discharge start before the EMG activity It can further be seen in Fig 6 that the correlation between the impulse frequency and the force was poor This was also a

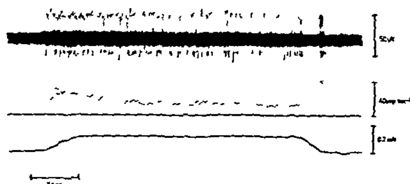


Fig 7 Afferent unitary activity during voluntary contraction showing discharge on relaxation. The subject made a contraction against a fixed resistance of the flexor muscles acting on his index. From above are shown the single unit impulses, the instantaneous impulse frequency and the torque due to the muscle contraction Straight line indicates zero impulse frequency

typical finding for the majority of the endings which were studied in this respect. It seems that a poor correlation between the impulse frequency and the active force militates against the notion that the ending was a Golgi tendon organ and consequently it is a support for the notion that the activity originated from a muscle spindle as these were the only two alternatives

The voluntary contractions supplied important clues for the classification of the endings also in another way. As shown by the example in Fig 7 an enhanced discharge appeared in some cases while the active force was decreasing when the subject relaxed after a sustained contraction. During this phase there was very likely a length increase of the intramuscular structures due to the pull by the series elastic elements. As this discharge appeared while the muscle force was decreasing and the length was increasing it seems justified to conclude that the discharge originated from an ending with a high dynamic sensitivity to changes of length i.e. a muscle spindle primary ending and definitely not from a Golgi tendon organ. The nerve discharge at the end of the contraction in Fig 7 was probably initiated in a similar way as the burst of impulses which are seen in group Ia afferents on the falling phase of an isometric twitch in animal experiments (e.g. Granit and van der Meulen 1962). Such a discharge on relaxation after a voluntary contraction without external shortening was exhibited by 14 units or 25 % of the endings tested with voluntary contractions

In contrast to the unit illustrated in Fig 6 there was for a few afferents a remarkably close relation between the unitary discharge rate and the active force during voluntary contractions. An example is shown in Fig 8. The instantaneous impulse frequency and the twisting force are illustrated when the subject contracted his flexor muscles acting on his fourth digit which was kept against a fixed resistance. When the record in A was obtained the dorsal flexion of the hand was 20° at the wrist whereas this angle was 30° when the records in B were obtained i.e. the muscles were slightly longer. The angles at the finger base joints were 140° and the

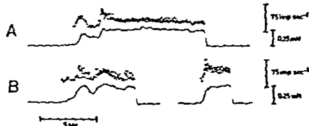


Fig 8 Single afferent unit discharge during voluntary contractions showing a pronounced correlation between the impulse frequency and the muscle force Upper traces show instantaneous impulse frequency, lower traces the torques due to the muscle contractions Zero impulse frequency at the lower end of the calibration bars The subject activated voluntarily the flexor muscles acting upon his fourth digit which was held against a fixed resistance The muscle lengths were slightly different in A and B as the hand was held in 20° dorsiflexion in A and 30° dorsiflexion in B

angles at the interphalangeal joints were 180° It is seen that the two variables the active muscle force and the impulse frequency, agreed very well, suggesting that the ending signalled the active force without any pronounced dynamic sensitivity This finding was regarded as a support for the notion that the ending was a Golgi tendon organ On the other hand, this ending hade some characteristics which are not typical of Golgi tendon organs It could be driven by vibratory stimuli although the maximal frequency was only 50 Hz and it responded with a short train of impulses of low frequency during a passive increase of the muscle length However these findings do not exclude the possibility that the discharge originated from a Golgi tendon organ (Alnaes 1967, Brown *et al* 1967)

The most employed method, in animal experiments to differentiate between muscle spindle and Golgi tendon organ afferents is to induce a maximal muscle twitch with an electric shock on the nerve This test has been found to be of no practical value in the present study, largely because the twitch inevitably resulted in a displacement of the recording electrode and the unitary discharge became indiscriminable This occurred even at small twitches far below maximal ones For the same reason it has not been possible to measure the conduction velocity of the single afferent fibres

Discussion

The purpose of the *present investigation was to study the basic functional characteristics* of slowly adapting muscle receptors in man The material was rigorously selected in order to exclude units of any other type The conclusion that the discharges were recorded from afferent fibres and not from efferent fibres rests upon the findings that the units exhibited prompt distinct and reproduceable responses to local deep pressure which was effective only within a small and well defined area for any one unit. Further there was no indication that the mechanical stimuli employed ever induced a discharge in α motor fibres which could possibly be mistaken for an afferent activity A number of findings proved that the receptors were located

in deep structures and not in the skin the unitary discharges were encountered when the electrode tip was in the portions of the nerves which are constituted of fibres distributed to deep structures (Sunderland 1945) as indicated by several signs (Vallbo and Hagbarth 1968, Hagbarth and Vallbo 1968 a and b), the mechanical thresholds for local pressures were high the unitary discharges were markedly affected by procedures which are not liable to have a large effect upon skin repectors but well upon intramuscular receptors such as voluntary contractions without changes of the muscle lengths and further, the majority of the units were recorded from the median nerve which does not innervate the skin area of the region in which the arm receptors were located It seems that these pieces of evidence allow the conclusion that all the unitary discharges studied were recorded from afferents having their receptors located in deep structures

Several types of deep receptor units were primarily excluded as constituting a notable portion of the material As all the endings were slowly adapting pacinif rm receptor units were not present in the material Joint receptors were excluded mainly because the endings were located far from the joints Further the reasonable assumption is made that the technique does not allow the recording of single unit activities in group III afferent fibres Consequently there remains a very limited number of possible receptor types which constitute the material One group are the units which have large diameter nerve fibres and slowly adapting receptors located outside the muscles and outside the joint regions Studies in animals indicate that there are on the whole very few such units Hunt and McIntyre (1960) found that approximately 10 % belonged to this group of the slowly adapting receptor units in the nerve to the flexor digitorum longus muscle and the interosseal membrane in the hindleg of the cat This branch of the peroneal nerve probably contains a comparatively large proportion of such units Further, many of these receptors are activated solely by particular types of mechanical stimuli which were not used in the present investigation such as squeezing the two bones in the leg together The other group of units which may constitute the present material are the slowly adapting intramuscular receptors with large diameter nerve fibres *i e* muscle spindle afferents and Golgi tendon organ afferents The above reasoning seems to justify the conclusion that more than 90 % of the units were of these types and it seems that the main lines of reasoning and the conclusions of the present report are not invalidated even if a few units with extramuscular receptors were included

More specifically the purpose of the present study was twofold One was to obtain a survey of the various types of unitary discharges encountered from intramuscular receptors in man and the other one was to experimentally penetrate the possibilities to activate these receptors and to modify their discharges This was pertinent as the experimental possibilities were markedly different from the possibilities in corresponding experiments on animals For a future more detailed analysis of the muscle receptor activity in man these two types of information seem to be of fundamental nature a general appreciation of the types of activities encountered and a knowledge of the test procedures which are useful and valuable In

animal experiments the separation of muscle receptors in Golgi tendon organs and muscle spindle endings is usually based upon the discharge during a maximal muscle twitch (Matthews 1933) This test has been vastly accepted although it does not always seem to give a correct differentiation of the endings particularly not in flexor muscles (Haase and Schlegel 1966 Green and Kellerth 1967) By definition the muscle spindle afferents are separated in group I and group II on the basis of their conduction velocities and there are clear functional differences between the endings of these two types of fibres (Cooper 1959 1961 Harvey and Matthews 1961, Appelberg 1962 Matthews 1963) However, the conduction velocity does not give a perfect separation according to the functional properties of the endings in the range close to the borderline between group I and group II conduction velocities Matthews (1963) pointed out that a test of the dynamic sensitivity of the endings to passive muscle stretch may give an adequate separation In the present study it was for technical reasons not possible to make use of these two testes in order to determine the receptor type electrically induced muscle twitches and conduction velocity measurements Therefore the classification had to be based upon other observations and test procedures Before considering in detail the findings which form the basis for the classification it seems relevant to consider in which respects the present material was a selected one and if so which are the likely implications concerning the constitution of the material As the experimental situation was mechanically delicate only weak mechanical stimulations and small passive joint movements were employed Therefore there was a selection of low threshold receptors and this very likely resulted in an over representation of muscle spindle afferents compared to Golgi tendon organs (Matthews 1933) Further there was probably an over representation of group Ia afferents before group II afferents as there is a greater chance to discriminate a unitary discharge from a larger fibre and besides it is often very difficult to find the receptive field of a secondary ending to local mechanical stimuli (Bianconi and van der Meulen 1963 Renkin and Vallbo 1964) It seems thus justified to assume that in the present material there was an over representation of group Ia afferents over Ib and group II afferents This selection is relevant for the evaluation of several test findings which provided supportive but not conclusive evidence for the notion that a repector was a muscle spindle primary ending

The tests which supplied the most significant evidence concerning the type of receptor *were passive joint movements when a pronounced dynamic sensitivity* could be demonstrated and voluntary contractions when a discharge appeared on relaxation It seems that either of these two findings were very strong evidence for the conclusion that the receptor was a muscle spindle primary ending Responses to vibration and tendon taps support the notion that the receptor was a muscle spindle primary ending as these procedures are essentially tests of the dynamic sensitivity An irregular discharge or a change of the impulse frequency while the experimental situation remained unaltered were interpreted as effects of intrafusal contractions and these findings were regarded as very strong evidence for the conclusion that the

afferent discharge originated from a muscle spindle. However, these findings do not allow a differentiation between group I and group II spindle afferents. A continuous discharge in relaxed muscles at intermediate muscle lengths seems to lend some support for the notion that the receptor was a muscle spindle ending although recent findings from animal experiments (Alnaes 1967) indicate that almost 20 % of the Golgi tendon organs in some muscles may be spontaneously discharging under similar conditions. Analysis of unitary afferent discharges during voluntary contractions offered some further possibilities to differentiate between Golgi tendon organ and muscle spindle afferents. The voluntary contractions were all done against a fixed resistance implying that there were no joint movements. However, it is generally recognized that, also under strict isometric conditions, there are some changes of the lengths of the intramuscular structures. These length changes were very likely more pronounced in the present study than in most animal experiments in which a single muscle tendon is rigidly connected to a force recording device and it was assumed that there was a considerable shortening of the intramuscular structures as the active force increased and a considerable lengthening as the active muscle force decreased. Some receptors were encountered which responded accurately to the twisting force under these conditions (Fig. 8). It was concluded that these endings were Golgi tendon organs. However, the majority of the endings exhibited a discharge rate which was not very closely related to the active muscle force. A poor relation in this respect is not unexpected from muscle spindle endings under these conditions, as the significant input variables determining the impulse frequency of a muscle spindle primary ending are muscle length, rate of change of muscle length and fusimotor activity. It was concluded that the afferents which exhibited a poor relation between the active muscle force and the discharge rate were muscle spindle endings. In another type of analysis, it could be demonstrated that in the steady state the impulse frequency of a unit was closely related to the muscle length but poorly related to the twisting force under active and passive conditions. This finding seems to be a strong support for the notion that the ending was a muscle spindle afferent and not a Golgi tendon organ.

Thus, a number of test procedures were useful in order to differentiate between the various types of intramuscular receptors. In the present material as many as 39 % of all the units could be classified as group Ia afferents by means of two simple tests: passive joint movements and voluntary contractions without external shortening. Even if the receptor type could not be determined for every unit it seems that the possibilities in general are satisfactory and with a further development of the technique a more complete classification will probably be possible.

A detailed analysis of the afferent discharge from muscle receptors in conscious man seems to be well worth while from the physiological point of view. This preparation provides a unique opportunity of studying the proprioceptive discharge during muscular activities which are governed by physiological processes in the highest motor centers of the brain and therefore a possibility of evaluating the role of the fusimotor system and the muscle spindles in voluntary contractions. In animal ex-

periments one may electrically stimulate the motor cortex in the anesthetized preparation, but it can not be taken for granted that such an analysis reveals the functional significance of the fusimotor system in voluntary contractions as pointed out by Koeze Phillips and Sheridan (1968) In a human subject the afferent activity can be analysed under a variety of conditions while complex motor activities are performed on instructions Voluntary contractions may easily be varied with regard to intensity, time course extent of movement fatigue and mental effort whereas in animal experiments the proprioceptive discharge associated with reflexes and respiration may be analysed motor acts which are comparatively invariable (*e g* Eldred Granit and Merton 1953 Critchlow and von Euler 1963)

In the present investigation the purpose was not to tackle specific physiological problems nevertheless some suggestions concerning the role of the muscle proprioceptor discharge emerge from the material The vast majority of the endings responded with a continuous discharge during voluntary contractions without external shortening (*cf* Hagbarth and Vallbo 1968 b 1969) As a large proportion of the receptors were muscle spindles endings this clearly shows that the fusimotor system is activated during voluntary contractions Only 13 % of the endings did not increase their discharge during voluntary contractions as used in the present investigation Considering that the contractions were far below the maximum it may be assumed that during stronger voluntary activations the afferent discharge from all the muscle spindles would increase implying that there are connections from the motor cortex to all of the muscle spindles in these muscles However there was very likely a selection in favour of endings which had low thresholds to local mechanical stimuli but it is not easy to evaluate the significance of this selection in this context In the vast majority of the tests during the voluntary contractions the afferent discharge started simultaneously with the EMG activity or slightly later The afferent activity was on the whole very precise in its onset and cessation and it coincided in time with the α motoneuron discharge as revealed by the EMG activity suggesting that the motor cortex exerted a very powerful control of the intrafusal muscular system Similar findings have been reported by Koeze Phillips and Sheridan (1968) on electrical stimulation of the precentral cortex in the baboon The present findings on this point which are in agreement with earlier reports (Hagbarth and Vallbo 1968 b 1969) provide no direct support for the notion that the impulses in group Ia muscle spindle afferents initiated the α motoneuron discharge through the spinal reflex connections (*cf* *Merton 1951 Eldred Granit and Merton 1953*)

One unit was found the discharge of which was very accurately related to the active force during voluntary contractions (Fig 8) As the contractions probably implied some length changes of the intramuscular structures it must be concluded that the ending had a low sensitivity to the muscle length and to the rate of change of muscle length On the other hand the ending was very sensitive to active muscle force but not to the rate of change of force These findings are strong supports for the notion that the receptor was a Golgi tendon organ The discharge characteristics of this ending during voluntary contractions are consonant with the notion that the

tendon organs during muscular activity "continuously provide information about the degree of active contraction in the muscle" (Jansen and Rudjord 1964)

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Dopamine-Containing Cells in Sympathetic Ganglia

By

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Abstract

BJORKLUND A L CEGRELL, B FALCK, M RITZÉN and E ROSENGREN *Dopamine containing cells in sympathetic ganglia* Acta physiol scand 1970 78 334—338

In addition to noradrenaline (about 1 3—1 5 μg/g) dopamine (about 0 2 μg/g) is present in the sympathetic chains of the cat and pig By means of a recently developed microspectrofluorimetric method, the cellular localization of these two catecholamines has been studied Dopamine was found in a special type of small intensely fluorescent cells ("SIF-cells) located among the adrenergic ganglion cell bodies Most of the dopamine present in the sympathetic ganglia is probably stored in these cells

With the histochemical fluorescence method of Falck and Hillarp (Falck 1962 Falck *et al* 1962 Corrodi and Hillarp 1963 1964) two distinctly different cell types displaying specific fluorescence have been demonstrated in sympathetic ganglia the cell bodies of the sympathetic adrenergic neurons and a type of small intensely fluorescent cells (SIF-cells) (Eranko and Harkonen 1965 Norberg Ritzen and Ungerstedt 1966 Jacobowitz 1967) The identity of the SIF-cells is not fully understood they possess properties similar to both chromaffin cells and nerve cells (cf Siegrist *et al* 1968) In microspectrofluorimetric studies Norberg *et al* (1966) demonstrated that SIF-cells store a primary catecholamine in the rat ganglia They could not decide however whether this amine was noradrenaline or dopamine Recently it has been possible to differentiate these two primary catecholamines by means of microspectrofluorimetry Bjorklund Ehinger and Falck 1968) taking advantage of the dehydration reaction of the noradrenaline fluorophore which occurs at acid pH Corrodi and Jonsson 1965 In the present investigation this method has been applied to the fluorescent cells of sympathetic ganglia in combination with chemical analysis

Material and Methods

Chemical determinations Part of the sympathetic chain cervical and thoracic division were dissected out from 11 pigs 1 animal for each determination and 19 cats 3 and 16 a was in two determinations see Table I Fluorimetric determinations of catecholamines (nor

adrenaline adrenaline and dopamine) were performed according to the method of Bertler *et al* (1958) as modified by Haggendal (1963)

Microspectrofluorimetric analyses Equivalent parts of the sympathetic chain to those used for chemical determinations from 5 pigs 7 cats and 12 rats were processed for fluorescence microscopy as described by Falck and Owman (1965) The tissues were freeze-dried and exposed to formaldehyde for 1 hr at +80° C The formaldehyde was generated from para formaldehyde equilibrated in air with about 50 % relative humidity The sections used for microspectrofluorimetry were gently deparaffinized in xylene and then exposed to the vapours from a concentrated HCl solution at room temperature according to Bjorklund *et al* (1968) In addition some sections were subsequently treated with the vapour from a concentrated NH_3 solution The measurements were performed by means of fluorescence microspectrophotometers as earlier described (Caspersson *et al* 1965 Ritzén 1967 Bjorklund *et al* 1968) The spectra were adjusted for systemic instrument errors (*cf* Ritzén 1967, Bjorklund *et al* 1968) Upon HCl treatment, the dihydroisoquinoline fluorophore of noradrenaline (ex- [illegible] max 330 nm), [illegible] non-quinoidal [illegible] When viewed [illegible] ecrease of the [illegible] pamine fluorophore is not notably affected

Results

The chemical determination showed that the sympathetic chains in pig and cat contain both dopamine and noradrenaline (see Table I) No significant amounts of adrenaline were detected Histochemically a strong fluorescence was seen in delicate varicose nerve terminals and a relatively weak fluorescence in the cytoplasm of the sympathetic adrenergic cell bodies Among the cell bodies or in the bundles of their axons SIF cells were found often located in clusters (Fig 1) as described earlier by Norberg *et al* (1966) Their number varied widely but they were found in most ganglia

TABLE I

	NA µg/g M±S E M (n)	DA µg/g M±S E M (n)
Symp chain pig	1 54±0 53 (11)	0 24±0 04 (11)
Symp chain cat	1 28 and 1 32	0 15 and 0 15

The concentrations of noradrenaline (NA) and dopamine (DA) in sympathetic chain of pig and cat

When examined in the fluorescence microspectrophotometer the cell bodies of the adrenergic neurons as well as the SIF cells showed excitation/emission spectra with peaks at 410/470 nm (Fig 2 and 3) identical with catecholamines in protein models or in other cell systems (Caspersson Hillarp and Ritzen 1966 Ritzen 1966, Norberg *et al* 1966 Bjorklund *et al* 1968) After a short exposure (about 15—60 sec) of the deparaffinized sections to HCl vapour, the excitation spectra of both types of cells changed in a way typical of catecholamines *i e* the peak shifted to about 370 nm (corresponding to a transformation of the fluorophore into the



Fig 1 Clusters of small inter fluorescent cells (SIF-cells) i sympathetic ganglion of the Note the difference in fluoresc intensity between the SIF- and the cell bodies of the sym thetic adrenergic neurons ×

quinoidal form) (Fig 2 and 3) In the SIF-cells this peak persisted during co tinued exposure to HCl vapour, whereas that of the adrenergic ganglion cells rap ly passed over to a very weak fluorescence, showing an excitation spectrum wit shoulder at around 320 nm (Fig 3) The excitation spectrum of the SIF-cells turned to the original after an additional exposure to NH_3 vapour In the standa fluorescence microscope no gross change in the fluorescence intensity of the SI cells was noted during the HCl treatment Thus, in the spectral recordings t fluorescence of the SIF-cells behaved like the dopamine fluorophore and the fluore cence of the adrenergic ganglion cells like the noradrenaline fluorophore (*cf* Björ lund *et al* 1968)

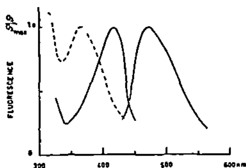


Fig 2

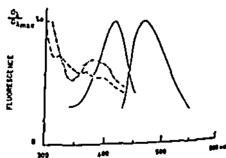


Fig 3

Fig 2 Excitation and emission spectra obtained from SIF-cells of the stellate ganglion of th cat, before (——— and after ( ) treatment of the section with *HCl vapour* Th excitation spectrum is characteristically changed showing one peak at 370 nm and one a 320 nm The emission spectrum did not change upon the treatment with HCl The amplitude of the curves do not represent fluorescence intensity

Fig 3 *Excitation and emission spectra from adrenergic ganglion* cell bodies before (——— for 2 min ( ) and 3 min (al change of the excitation maximum from At the same time the fluorescence intensity decreases to a much greater extent than that indicated in the figure

After very intense treatment of deparaffinized sections with HCl vapour (5 min or more), the excitation spectra of both types of cells changed once more displaying an unidentified and possibly unspecific product with an excitation maximum at 430—440 nm This was not accompanied by any simultaneous change in emission spectrum After such an "over exposure" to HCl adrenergic noradrenaline containing cell bodies showed a considerable fluorescence, and might be mistaken for dopamine containing cells when seen in an ordinary fluorescence microscope Such errors are avoided if the sequence of decrease and reappearance of the fluorescence in a series of increasing HCl treatments is followed

Discussion

Laverty and Sharman (1965) found both dopamine and noradrenaline in sympathetic ganglia of some species, including cat Their results agree with those obtained in this investigation where dopamine and noradrenaline have been demonstrated in the sympathetic chain of cat and pig In fact the microspectrofluorimetric analyses showed that the fluorescence in the SIF cells in the sympathetic ganglia in these animals and in the rat behave like that of the dopamine fluorophore upon HCl treatment It cannot be excluded, however that the SIF-cells in the species studied store noradrenaline besides dopamine as a simultaneous occurrence of these two amines cannot be evaluated with the available method In experiments with protein models containing mixtures of dopamine and noradrenaline it has been found that dopamine can be identified microspectrofluorimetrically if the concentration of dopamine is 10 % or more of the noradrenaline concentration (Baumgarten and Björklund unpublished) This means that only very low concentrations of dopamine can be present in those structures showing the characteristics of noradrenaline Thus most of the dopamine in sympathetic ganglia seems to be located in the SIF cells

Small intensely fluorescent cells similar to those described in this paper have been described also in other tissues Owman and Sjöstrand (1965) found such cells in the male accessory genital organs of some species They suggested that these cells store mainly adrenaline in the dog and monkey In preliminary microspectrofluorimetric analysis of the small intensely fluorescent cells in the dog prostate (Björklund and Cegrell unpublished) these cells showed the characteristics of dopamine which suggests the presence of this amine also in these cells SIF cells have also been found in the heart (Jacobowitz 1967, Ehinger *et al* 1968) and in the pancreas (Alm *et al* 1967) sometimes also within non adrenergic presumably cholinergic ganglia

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Acta physiol scand 1970 78 339—346

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Transmembrane Transport of Chloride and Iodide in Proximal Rat Tubules

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Abstract

DANIELSON B G, E PERSSON and H R ULFENDAHL *Transmembrane transport of chloride and iodide in proximal rat tubules* Acta physiol scand 1970 78 339—346

The transport mechanism for chloride across the proximal tubular epithelia in the rat was investigated with a free flow micropuncture technique The purpose of the investigation was to determine whether there is an active carrier system for chloride transport out of the tubule with different affinities for different halides and higher for iodide than for chloride The TF/P values for sodium potassium chloride and iodide were determined simultaneously The TF/P value for chloride (1 15) was significantly lower than the TF/P value for iodide (1 73) It was concluded that there is no evidence of an active transport system and that chloride and iodide are transported passively out of the tubular lumen the permeability for iodide being somewhat lower than for chloride

The reabsorption of the large volume which takes place in the proximal tubules is commonly regarded as depending on an active transport of sodium ions out of the tubular lumen to the interstitial fluid Chloride and to a certain extent bicarbonate ions are reabsorbed and are regarded as passively following sodium ions Water passively follows the salt transport without any measurable osmotic gradient between tubular lumen and plasma so that the urine within the proximal tubules is always isotonic An excellent review is given by Windhager (1968)

There has been much debate as to whether chloride transport is active or passive From clearance experiments Kruhoffer (1950) and Saugman (1957) concluded that chloride was transported passively and there was a mutual competition between small anions in which the physico-chemical properties determined the amount of reabsorption Results which accord with this view have also been reported by Walser and Rahill (1965 a b 1966 a, b)

Giebisch and Windhager (1963) found no evidence for active reabsorption of chloride in the proximal tubules of Necturus but concluded that there was a

mediated transport process from plasma into the tubular lumen. In experiments wi determination of the electrical potential and the concentration differences betwee tubular lumen and plasma Kashgarian *et al.* (1963) and Kashgarian, Warren ar Levitin (1965) found evidence of a force which moves chloride into the tubul lumen. Fromter and Hegel (1966) performed similar experiments under conditio where the electrode position inside the lumen was carefully checked and found n evidence for an active transport of chloride.

In the present investigation we have tested the assumption of a carrier system wi different affinities for different halide ions in the proximal tubules in the rat. Eithe a net active transport mechanism or exchange diffusion could establish differe tubular fluid to plasma concentration ratios (TF/P), for different halides. Th existence of such a transport system has been shown in the stomach by Heinz, Obrin and Ulfendahl (1954) and Davenport (1943). With a free flow micropunctu technique we have determined the chloride and iodide TF/P values in proximal ra tubules.

Methods

Operation and sampling technique

The experiments were performed on male albino rats of the Sprague Dawley strain weighin between 200 and 350 g. Before the experiments the animals were allowed free access to foo and water. Anesthesia was induced with an intraperitoneal injection of 1 % solution of chlora lose (150 mg/kg b.w.). The body temperature was controlled and regulated with an intrarecta contact thermometer and a heatable operating table.

All animals were tracheostomized. Catheters were placed in the right jugular vein and the left common carotid artery. The left kidney was exposed through a paramedian incision. The kidney was fixed with cotton wool and a Perspex ring. Good fixation made sampling over a period of 10–15 min easy. The sampling Pyrex pipette had an outer diameter of 10 μ and were ground on an air driven grinding machine. The pipette was inserted into a proximal tubule which had been first identified by the passage of lissamine green after an i.v. injection (Stein hausen 1963). The sampling was performed with a motor driven perfusion syringe arranged for suction modified as according to Sonnenberg and Deetjen (1964). The sampling pipettes were filled with mineral oil (liquid paraffin).

A second i.v. injection of lissamine green was given during sampling in order to demonstrate a free flow around the cannula. Plasma samples were taken at the same time as the sample from tubular fluid. The arterial blood pressure was recorded continuously during the experi ments. 15 min before the sampling 1–2 mCi I^{131} iodide was injected i.v. In some cases 0.1 ml of a 0.150 M sodium iodide was injected i.v. as a carrier.

Analysis

After removal from the tubule the sample was transferred immediately to the bottom of a small glass cup filled with mineral oil. The surface of the glass cup had been previously treated with Siliclad® (Adams) so as to prevent the droplet spreading on the glass surface. The sample had a volume of between 1.1 and 3.0 nl and drops of this dimension can be stored in mineral oil for several hours without any volume changes (Hellman, Ulfendahl and Wallin 1967).

1. *Sodium* and *potassium* concentrations were analyzed simultaneously with an integrating flame photometer. The coefficient of variation could be as low as ±2–4 % (Oberg, Ulfendahl and Wallin 1967). The volume used for each determination was about 0.5 nl. As a rule the analysis was made in duplicate or triplicate.

2. The *chloride concentration* was analyzed electrochemically according to the method of Ramsay, Brown and Croghan (1955). The samples were taken with a constant volume pipette with a volume of about 0.5 nl. See Fig. 1. The coefficient of variation for 15 standard samples of chloride with a concentration within the range used in this work was ±1.2 %.

3. I^{131} analysis was performed in a Packard Tricarb (314 EX) or Beckman liquid scintilla tion counter (CPM 200). The volumes used for the analysis were between 4 and 15 nl taken

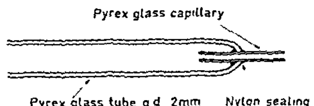


Fig 1 The picture shows the constant volume pipette used for taking samples from drops stored under oil The "constant volume" capillary was fixed to the large tube by a nylon seal The Pyrex capillary must be thin walled to obtain the highest accuracy when taking samples from small drops of water in the oil phase The outer surface was treated with Siliclad® (Adams) The pipette is filled with oil (50 % liquid paraffin and 50 % Silicone oil MS 2 centistoke) This oil composition made the immersed pipette easily visible

with a constant volume pipet (Fig 1) The analysis was made in duplicate At least 3000 counts above background were counted The volume of the scintillation liquid was 13 ml and

azole), 0 12 g Dimethyl

±6 % with a concentra

Calculations

Results

The results are summarized in Table I Like other authors we found a TF/P value for sodium close to unity in free flow conditions (Kashgarian *et al* 1963 The TF/P value for potassium was 0 77 a value which seems dependent upon the metabolic and acid base status of the animal (Giebisch and Windhager 1964) The TF/P value found by Kashgarian *et al* (1963) for chloride is close to our calculated value of 1 15

The TF/P value for chloride was significantly different from that for iodide by Student t test in which each experiment was its own control ($P < 0.001$ The TF/P values for chloride and iodide did not change in the presence of carrier sodium iodide The ratio TF/P chloride/TF/P iodide is a measure of transport rate differences between the two halides out of the proximal tubular lumen A value less than unity means a lower transport rate of iodide out of the tubule compared with chloride ions During all experiments the arterial blood pressure was never below 90 mm Hg

Discussion

The method of comparing the transport of ions which seem to be transported by the same carrier system has been used by other investigators in other organs (Heinz Öbrink and Ulfendahl 1954 Davenport 1943 The validity of such a comparison in the kidney must however be discussed

TABLE 1 Electrolyte concentrations in proximal tubular fluid during free flow conditions a plasma

	Means	S D	n
Na_{TF} mM	140	±16	10
Na_P mM	132	±16	11
$\frac{TF}{P}$ Na	1 06	± 0 18	8
K_{TF} mM	4 0	± 0 7	10
K_P mM	4 7	± 0 8	11
$\frac{TF}{P}$ K	0 77	± 0 04	7
Cl_{TF} mM	134	±11	19
Cl_P mM	118	± 9	19
$\frac{TF}{P}$ Cl	1 15	± 0 12	19
$\frac{TF}{P}$ I	1 73	± 0 34	14
$\frac{TF/P\ Cl}{TF/P\ I}$	0 69	± 0 12	14

Index TF means proximal tubular urine and index P is plasma

The experiments in this work were made under free flow conditions The punctur site was not identified but it can be safely assumed because of the random puncturin that in most experiments at least the puncture site was sufficiently distant from th lomerulus for a net outflow of chloride and iodide to have occurred before th sampling point If there is an active transport mechanism for chloride and iodide ou of the tubules similar to that occurring into the stomach a higher affinity for iodid than for chloride ions would give a ratio for TF/P chloride/TF/P iodide greater tha unity In our experiments the ratio was significantly lower than unity a result whicl suggests that halide ions are not transported actively out of the tubule

This approach has been made indirectly by several authors who have related dif ferent halide clearances to chloride clearance Thus Kruhoffer (1950) found that the assumption of a passive proximal reabsorption of easily diffusible ions in competition with one another well agreed with a number of clearance investigations. Sauerman (1957), who made clearance determinations of halides concluded there was a passive transport mechanism for chloride in the proximal tubule

Walser and Rahill (1965 a also determined the iodide and chloride clearances and found no evidence for active chloride transport but concluded that chloride and iodide were passively reabsorbed and that this occurred in coextensive regions in the case of normal urinary chloride excretion The same conclusions were drawn from later experiments (Walser and Rahill 1965 b 1966 a b) where they compared

nitrate thiocyanate perchlorate bromide and fluoride clearances with the chloride clearance The drawback with clearance investigations is the difficulty in determining transport in different parts of the kidney

With stop flow experiments Kiil and Aukland (1962) found that iodide probably had a lower tubular permeability than chloride Also Williamson *et al* (1962) found with stop flow experiments a lower permeability for iodide than for chloride, and the results were interpreted as being due to a passive reabsorption of iodide in the proximal tubules of dogs

Using the flux ratio equation of Teorell (1949) and Ussing (1949) Giebisch and Windhager (1963) obtained results indicating a passive transport of chloride in the proximal tubules of Necturus However, they also found evidence for some carrier mediated process either exchange diffusion or secretory movement into the tubular lumen which Kiil and Aukland (1962) considered unlikely From studies of the short circuit current across the proximal tubular wall Giebisch *et al* (1964) concluded that there was an active sodium transport large enough to explain the fluid reabsorption in the proximal tubule of the rat However serious doubts exist as regards the application of the short-circuit method to the kidney which has been analyzed critically by Windhager and Giebisch (1965)

Kashgarian *et al* (1963) injected into the tubules solutions of raffinose which were reabsorbed slowly After sufficient time to establish a constant concentration of the electrolytes they then simultaneously measured the concentration difference for chloride and the electrical potential over the proximal tubular membrane A steady state condition with a small net reabsorption was established They further assumed that the small reabsorption could be neglected and applied the work equation of Ussing (1960) i e

$$E_{Cl} \quad \frac{RT}{zF} \quad \ln \frac{C_i}{C_o} + E + \frac{RT}{zF} \quad \ln \frac{M_i}{M_o}$$

where E_{Cl} active chloride potential the C s = concentration within and outside the tubules the M s are the mass flows into and out of the tubule E = electrical potential difference between tubular lumen and interstitium They obtained a value for an active chloride transport potential of 23 mV a force which moved chloride into the tubular lumen The authors however expressed their own doubts about the result of this experiment Later Kashgarian Warren and Levitin (1965 repeated these experiments with another substance the macromolecular polyvinyl pyrrolidone (PVP) injected into the tubular lumen The advantage with PVP was that no net volume flow could be detected out of the tubule They proposed that there was a force which pumped chloride into the tubular lumen They also supposed that the chloride secretion pump should be linked to the acidification process of the urine in the proximal tubule

Fromter and Hegel (1966 subjected the potential measurements to a critical analysis With careful checking of the position of the electrode they could not detect any potential difference between the tubular lumen and the interstitium If the po-

tential was zero in an equilibrium condition they calculated that the active chloride transport potential should also be zero They then concluded that there was no evidence for active chloride transport across the proximal tubules Burg *et al* (1968) using isolated rabbit renal proximal tubules, were also unable to detect a potential difference although they obtained evidence for an active transport of sodium chloride out of the lumen Abramow Burg and Orloff (1967) studied the distribution of chloride in experiments on suspensions of rabbit renal tubules and on fragments of single proximal tubules They concluded that there was evidence suggesting that the chloride transport in the kidney tissue is not a simple passive process It is however difficult to draw any conclusions on the nature of the transtubular chloride transport from their experiments

In our experiments it seems probable that the degree of fluid reabsorption varied considerably because of the random position of the puncture site in the proximal tubules The scatter of our TF/P chloride and TF/P iodide values is however fairly small indicating that a steady state existed for these partition ratios The result therefore indicates that a higher transtubular concentration difference for iodide than for chloride would be needed to obtain the same halide flux over the proximal tubular epithelium The necessity for a greater concentration gradient for iodide ions may depend on

1) a lower permeability for iodide ions than for chloride ions
2) an active transport mechanism for halide ions which transports iodide at a higher rate than chloride into the tubular lumen

This latter transport would presumably exist as a carrier system with a higher affinity for iodide than for chloride In our opinion the first hypothesis seems to be the more probable even if it is impossible to exclude the second alternative The idea of a carrier system with a higher affinity for iodide than for chloride seems to have some support in the work of Walser and Rahill (1965 a These authors found in experiments with low chloride excretion that the excretion fraction for iodide was lower than that for chloride In experiments with normal chloride excretion conditions however the excretion fraction for iodide was always higher than that for chloride Other experimental support has been provided for the existence of an active chloride reabsorption in rat distal tubules in the case of low chloride excretion (Rector and Clapp 1962)

In a short communication Baumann *et al* (1966) reported measurements of the outflow of Cl^{36} and I^{131} from the rat proximal tubules They found a half time for the chloride[36] outflow of 0 88 sec and for iodide[131] 1 38 sec The outflow rate for chloride exceeded that for iodide quite in accordance with our result From their result it can be calculated that if the chloride concentration in a proximal tubule exceeded that outside the tubule by 20 mM this would be sufficient to give a passive outflow of chloride as large as that of sodium In our experiments we found a concentration difference of about 15 mM for chloride in agreement with the results of Baumann *et al* (1966) A calculation can also be made (Kashgarian *et al* 1963) of what the tubular bicarbonate concentration should be with the chloride concentra

tion actually found in the tubules Kashgarian *et al* (1963) calculated a value for intratubular bicarbonate concentrations of about 10 mM, which should correspond to a pH of 7 According to the results of Rector, Carter and Seldin (1965) and Vieira and Malnic (1968), this calculation is a good estimate The pH observed was slightly below 7 (6 7—6 8) If our conclusion of a passive reabsorption of chloride is correct the question of the reabsorption mechanism of bicarbonate ions then arises The result of Rector, Carter and Seldin (1965) supports the idea of an active hydrogen ion secretion into the tubular lumen Such a secretion would presumably result in conversion of some bicarbonate to carbonic acid and to CO which would be transported rapidly out of the tubule As yet it has still not been possible to determine quantitatively the permeability of the proximal tubules for bicarbonate ions Bank and Aynedjian (1967) reported results which suggest some transport of bicarbonate ions through the tubular walls Assuming a variable and higher permeability for bicarbonate ions than for chloride ions this might be a regulatory mechanism for the acidification of the proximal tubular urine, working together with or without an active secretion of hydrogen ions

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The Transport of Halide Ions across the Membrane of Distal Rat Tubules

By

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Abstract

Danielson B G E Persson and H R Ulfendahl *The transport of halide ions across the membrane of distal rat tubules* Acta physiol scand 1970 78 347—352

The transport mechanism for chloride was studied in rat distal tubules with a free-flow micropuncture technique The tubular fluid to plasma concentration ratio (TF/P) was determined simultaneously for sodium potassium chloride and iodide The experiments were designed to determine whether there is a common active carrier mechanism for halide transport out of the distal tubules with a higher affinity for iodide than for chloride If such a transport system exists it should give rise to a lower TF/P value for iodide than for chloride. However the TF/P value for iodide was 0 78 and was significantly higher than for chloride (0 39) a result which is interpreted to mean that no such active carrier transport mechanism for halides exists in the distal tubules. In our opinion the differences in TF/P values depended on a lower passive permeability for iodide than for chloride

Studies of transmembrane transport of water and ions in the distal tubules may be complicated to perform This depends partly on technical difficulties and partly on physiological variabilities in the transport mechanisms Thus there are changes in water permeabilities (Ullrich Rumrich and Fuchs 1964) and there are also possibilities of different functions in different parts which for example is the case with potassium transport (Giebisch Klose and Malnic 1966) This may explain why the relatively few published micropuncture studies on halide transport in the distal tubules have given rise to controversy about the halide transport mechanism Kashgarian *et al* (1963) found no evidence for active chloride transport in the distal tubules Rector and Clapp (1962) measured potential and chloride concentration differences across distal tubular epithelia during massive osmotic diuresis induced with sodium sulfate and contrary to Kashgarian *et al* (1963) found that there was an active reabsorption of chloride in the distal tubules By comparison of excretion fractions of halides Walser and Rahill (1965 a b 1966 a b) also found in experiments with low chloride excretion evidence for an active transport of halides In stop flow experi-

ments on salt depleted dogs during sodium sulfate diuresis Schafer, Vander and Brubacher (1966) found no convincing evidence for an active chloride transport even though they could not definitely exclude such a mechanism

In a previous work (Danielson, Persson and Ulfendahl 1969) we have studied the transmembrane transport of chloride and iodide in proximal rat tubules These investigations were based on the hypothesis that the same active transport mechanism or possibly an exchange diffusion mechanism should transport all halide ions and that such a mechanism should have a higher affinity for iodide than for chloride ions Such a hypothesis is valid for the stomach, as has been shown by Heinz, Öbrink and Ulfendahl (1954) and Davenport (1943) This paper reports the estimation from free flow micropuncture experiments of the ratios of tubular fluid/plasma concentrations (TF/P) for chloride, iodide, potassium and sodium

Methods

Operation and experimental procedure

The experiments were performed on Inactin® anesthetized albino rats of the Sprague Dawley strain, weighing between 200 and 350 g The animals were allowed free access to food and water until the start of the experiment The Inactin® was given intraperitoneally as a 2 1/2 % solution in NaCl (154 mM) (150 mg/kg body weight) In order to keep the animals in a good general condition the body temperature was maintained at 38° C with the aid of a heatable operating table controlled by an intrarectal contact thermometer All animals were tracheostomized Catheters were inserted into the left jugular vein and the right common carotid artery

The left kidney was exposed through a paramedian incision and fixed with cotton wool and [illegible]

[illegible] berg and Deetjen (1964) Micropuncture was made under free flow [illegible] tubules were visualized by an intravenous injection of lissamine green Small amounts of this dye were also injected intravenously at intervals in order to check the patency of the tubules [illegible] rate of approximately [illegible] The mean blood [illegible] experiment [illegible] without, or in some cases with 0.1 ml of 0.150 M sodium iodide as a carrier After the collection the samples were treated as described in an earlier work (Danielson Persson and Ulfendahl 1969)

Analysis

1 Sodium and potassium were analysed in an integrating flame photometer See a previous work (Danielson *et al* 1969) The determinations were made in duplicate or triplicate with a volume of about 0.5 nl at each determination

2 Chloride was analysed electrometrically as according to Ramsay, Brown and Croghan (1955) See Danielson *et al* 1969)

3 I^{125} iodide was analysed by liquid scintillation in a Beckman liquid scintillation counter (CPM 200) See Danielson *et al* 1969)

4 The osmolality was determined in a microcryometer as according to Ramsay and Brown (1955)

Calculations

[illegible] total plasma [illegible] s 1.02 for [illegible] cording to [illegible] sma iodide [illegible] estigation

TABLE I Electrolyte concentrations in distal tubular fluid during free flow conditions and plasma

	Mean	S D	n
Na_{TF}	54	±26	18
Na_P	139	± 7	19
$\frac{TF}{P}$ Na	0 39	± 0 19	18
K_{TF}	7 9	± 2 0	9
K_P	5 1	± 0 5	11
$\frac{TF}{P}$ K	1 8	± 0 51	7
Cl_{TF}	45	±20	18
Cl_P	116	± 8	18
$\frac{TF}{P}$ Cl	0 39	± 0 18	18
$\frac{TF}{P}$ I	0 78	± 0 32	18
$\frac{TF/P\ Cl}{TF/P\ I}$	0 57	± 0 29	18

The sodium potassium and chloride concentrations are expressed in mmoles/l Index P means plasma and index TF means distal tubular fluid

Results

The results are presented in Table I The TF/P value for sodium was 0 39 and is somewhat higher than those values found by Kashgarian *et al* (1963) while the TF/P value for potassium was 1 8, in agreement with the work of Giebisch, Klose and Malnic (1966) Since the exact site of puncture was not known it may be misleading to compare TF/P values for potassium with those of other workers, as it is known that these values rise along the distal tubules in normal rats in both free flow and near zero net flux conditions (Giebisch, Klose and Malnic 1966) The value of 0 39 for TF/P chloride is somewhat higher than that obtained by Kashgarian *et al* (1963), but is significantly ($P<0.001$) lower than the value of 0 78 for iodide in the present study The test of significance used here was Student s t test with every experiment as its own control The (TF/P Cl)/(TF/P I) ratio is a measure of transport rate differences between the two halides out of the tubular lumen The ratio 0 57 is not significantly different from that obtained in the proximal tubules (Danielson *et al* 1969) In some experiments 0 1 ml of 0 15 M sodium iodide was injected i v as a carrier but the TF/P chloride and iodide were not different from when I^{125} was carrier free Walser and Rahill (1965 a) also reported no difference between nonradioactive and radioactive carrier free iodide clearances The volumes and osmolalities of the urine from each kidney were measured in some of the ex-

periments The osmolality varied between 500 and 2000 mOsm/kg There were r major differences between the two kidneys with regard to excreted volumes an osmolalities

Discussion

In an earlier investigation (Danielson *et al* 1969) we determined the relative tran port rates out of the tubule for iodide and chloride in proximal tubules The prese investigation had a similar aim We began with the hypothesis that if there is carrier system for chloride out of the distal tubules it will also transport iodide at higher rate Such an assumption was found to be valid for the stomach (Hein Öbrink and Ulfendahl 1951 and Davenport 1943) If such an active carrie mechanism for outward transport exists the result should be a (TF/P Cl)/(TF/P I ratio greater than unity in both free flow and stop flow experiments We found o the contrary a ratio significantly lower than unity in free flow experiments It th seems that the hypothesis for an active outward transport involving a carrier with higher affinity for iodide than chloride is almost certainly wrong

Kashgarian *et al* (1963) determined simultaneously the electrical potential an the TF/P chloride and by using the work equation of Ussing (1960) they cal culated that there was no active transport potential for chloride More recentl Fromter and Hegel (1966) from potential determinations confirmed the result o Kashgarian *et al* (1963)

Rector and Clapp (1962) claimed that they had shown an active transport out o the distal tubules These authors induced sodium sulfate diuresis in salt deplete rats In free flow micropuncture they determined the TF/P chloride and the trans tubular potential The highest measured potential was 120 mV (lumen negative) and using the Nernst equation

$$E = \frac{RT}{zF} \ln \frac{Cl_P}{Cl_{TF}}$$

they calculated the lowest chloride concentration which could be obtained if chloride was entirely passively transported out of the tubule They obtained a value of 1 mM for the chloride concentration but in some experiments the measured chloride concentration was much lower They concluded therefore that there was an active outward transport of chloride in the rat distal tubules This interpretation can be questioned for the following reasons 1 The measurements were performed during free flow conditions that is in some sort of steady state whereas the equation is only strictly valid for an equilibrium The effect of the outward bulk flow on the ion distribution was disregarded and this has been shown by Teorell (1953) to be of importance 2 The animals were salt depleted and the experiments were made under massive osmotic diuresis If an active transport mechanism for chloride exists during these conditions it is disputable whether it has any importance during normal conditions Even if there is an active driving force for chloride the capacity of the transport system may be small and not easily measurable with flux experiments

We have confirmed that the method used for the determination of low chloride concentration as used by Rector and Clapp (1962) is very accurate

Walser and Rahill (1965 a, b 1966 a, b) compared the clearances of iodide bromide and fluoride with that of chloride They expressed the relative transport rate out of the tubular lumen as

$$\frac{\log \text{excretion fraction for halide}}{\log \text{excretion fraction for chloride}} = k$$

They state that this k should be constant in different experimental conditions if both halides were passively transported out of the same segment of the nephron They found that k was constant for all halides under normal chloride excretion conditions and drew the conclusion that all the halides were transported out of the tubular lumen passively and coextensively The authors assumed as proposed by Rector and Clapp (1962) for rats that a TF/P Cl less than 0 01 meant an active transport of chloride At extremely low urinary chloride concentrations the urine concentration/plasma concentration ratio (U/P) of bromide and iodide but no fluoride were less than 0 01 They concluded that bromide and iodide were actively transported out by the same mechanism as chloride Normally the excretion fraction for iodide was higher than that for chloride but in the case of a very low chloride excretion rate the excretion fraction for iodide in some cases was lower This supports the concepts of Rector and Clapp (1962) regarding active transport and also suggests that our hypothesis of a carrier transport system out of the tubular lumen with a higher affinity for iodide may be correct in low chloride excretion With stop-flow and slow flow techniques Schafer, Vander and Brubacher (1966) studied different anion concentration gradients in distal tubules of the dog using gradients of SCN Cl and Br which were not significantly different from each other From their results they concluded that it is unlikely that any single active transport mechanism or three individual mechanisms could establish the same transtubular steady state concentration gradient The U/P ratio for iodide was always higher than that for chloride which indicated a lower permeability for iodide than for chloride The excretion pattern was not altered by salt depletion as found by Rector and Clapp (1962) in rats The Nernst equation was used by Schafer Vander and Brubacher (1966) to determine the potential from the U/P ratio assuming equilibrium conditions in the distal tubules The calculated potential was compared with that found in distal tubules in rats In control dogs the calculated potential was 60 2 mV and that measured by Rector Clapp and Seldin (1962) in rats was 53 8 mV In salt depleted dogs with sodium sulfate diuresis the calculated potential was 102 5 mV and the potential found in rats 73 0 mV If conclusions can be drawn from these comparisons they are suggestive of an active chloride transport but without having measurements of the potential in dog distal tubules the interpretations must remain questionable

Although an active outward transport of chloride may exist it seems to have a small capacity This mechanism seems to become manifest when the intratubular

chloride concentration is low, but has no major influence on chloride reabsorptio under normal chloride excretion patterns We consider that the observations of higher intratubular concentration of iodide than chloride may be explained by lower tubular permeability for iodide, as was also proposed by Walser and Rahi (1965 a 1966 b) and Schafer Vander and Brubacher (1966) Finally, it may b mentioned that Kiil and Aukland (1962) found evidence in stop-flow experimen of a lower passive permeability for iodide than for chloride Our conclusion is that is probable that halide transport is at least mainly passive in the normal rat dist tubules

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Water Permeability in Rat Proximal Tubules

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Abstract

PERSSON E and H R ULFENDAHL *Water permeability in rat proximal tubules* Acta physiol scand 1970 78 353—363

The diffusional w
periments with tri
or a net water flux
was 564 10^{-5} cm
flux is discussed
The large differences between osmotic water permeability and diffusional water permeability in zero net flux conditions cannot be accounted for entirely by an unstirred layer effect and are interpreted to mean that water transport along osmotic gradients is mainly a bulk flow through aqueous pores and not a diffusional flow and that the proximal tubular wall is a highly porous membrane

During the last years great interest has been centered on the magnitude and regulatory mechanism of the fluid reabsorption in the proximal tubules The active reabsorption of sodium is generally regarded as the driving force for the fluid reabsorption There is massive experimental support (Schatzmann Windhager and Solomon 1958 Giebisch *et al* 1964 Fromter and Hegel 1966) in favour of an active sodium transport The transport mechanism for the major anion chloride is more uncertain in view of the apparently contradictory experimental results (Kashgarian *et al* 1963 Fromter and Hegel 1966) Water transport is usually regarded as passive and following the ionic movement without any measurable osmotic gradients between tubular lumen and plasma so that the urine within the proximal tubules is always isotonic

The hydraulic water permeability in the proximal tubules was measured with osmotic gradients by Ullrich Rumrich and Fuchs (1964) The osmotic water permeability (Lp) found was low compared to the water flux which occurs with a normal transtubular osmotic concentration gradient Thus the mechanism for isotonic water reabsorption in proximal tubules is however still unclear The present investigation in which water permeability of the proximal tubules was measured with tritiated water was made in an attempt to clarify the water transport mechanism to some extent

Methods

30 male albino rats of the Sprague-Dawley strain, weighing between 200 and 300 g, were use The rats were fed on standard pellets (Ewos, Sodertälje, Sweden) and were allowed fr access to food and water until the experiment Anesthesia was induced and maintained b intraperitoneal administration of Inactin®,[1] 150 mg/kg b w, in a 0 154 M sodium chlori solution *Because of the inability of small animals to regulate their body temperatures durin* anesthesia a thermistor-thyristor-thermostat for continuous regulation of body temperature wa used (Persson, Ståhlbrandt and Ulfendahl 1969)

diameter of 8—10 μ with an air-driven grinding machine as according to Vurek *et al* (1967) The microperfusion was performed as according to Sonnenberg and Deetjen (1964) An oi droplet was injected into a loop of a proximal tubule, and a tubule with two more cortical loop distal to the puncture site was seltected for perfusion experiments The next loop wa punctured with a cannula connected to a microperfusion pump The part of the tubule be tween the first and the second puncture site was filled with mineral oil from the first cannula This cannula was then withdrawn and used to puncture the third cortical loop With th microperfusion pump a flow varying between 10 and 35 nl/min could be obtained With th cannula in the third loop samples were carefully removed at a low and constant rate of 1— nl/min during 2—5 min

tested by measuring the proximal

of lissamine green according to S

mined in one of two alternative ways, either directly with an ocular scale during the perfusion or by measurement of a neoprene cast The length of the perfused nephron part was always chosen to be longer than 130 μ, depending on the difficulty of accurate measurement of short distances In some cases the length was as long as 1000 μ The radius of the perfused tubule was 10 μ

Three different perfusion solutions were used

1 Isotonic solution The sodium concentration of this solution was a modified value of the concentration which Gertz (1963) calculated for the equilibrium value in the proximal tubule The weight composition of the solution was NaCl 100 mM, KCl mM, mannitol 90 mM, Cl inulin (33 g/l, 100 μCi/ml), THO 1 25 mCi/ml Since only a small volume of this solution was prepared the ionic concentrations and osmolality were measured by the same micromethod as used for the perfused samples The following values were obtained, Na 92 mM, K 1.3 mM Cl 107 mM, osmolality 326 mOsm/kg It must be noted that the total anion and cation con centrations are not the same

2 Hypotonic solution This hade the same nominal ionic, inulin and THO concentrations as solution (1) The mannitol concentration was, however, 60 mM The ionic concentrations obtained from micromeasurements were Na 100 mM, K 0 8 mM and Cl 110 mM and the

NaCl was 150 mM while the concen solution (1) The values obtained in mM and osmolality 410 mOsm/kg l oil (Hellman, Ulfendahl and Wallin

1967)

Analysis

with an integrating flame photometer The coefficient e can be as low as ±2—4 % according to the con 967) The errors obtained here were, however, some what greater The volume used for each determination was 0 5—1 nl

method developed by Ramsay, Brown and determinations within the range

to Ramsay and Brown (1955)

[1] Chem Fabrik, Promonta, GmbH, Hamburg

The coefficients of variation for the osmolality determinations within the range used in this paper were less than $\pm 3\%$ (n=4)

4 THO and C^{14} inulin (Inulincarboxy C^{14}, Calbiochem, Los Angeles, USA) were analyzed in a Packard tri carb 314EX liquid scintillation counter

The sample volume varied between 1 and 5 nl was taken with constant volume pipets (Danielson Persson and Ul

tillation fluid were added

PPO (2,5 diphenyloxazole)

Benzene) and 5 ml ethanol

ground were counted The

than $\pm$ 6 % of the mean value

Calculations

The two following equations were used for the calculation of the diffusional water permeability for THO (Persson 1969 b, Grantham and Burg 1966)

$$P_D = \frac{\O}{A} \ln \frac{C_{tub}(l_o)}{C_{tub}(l_s)} \tag{1}$$

where

P_D = permeability for THO (cm/sec)
$\O$ = volume flow through the nephron (cm^3/sec)
A = area of the perfused tubule (cm^2)
$C_{tub}(l_o)$ = THO concentration in the perfusion fluid (before perfusion) (cpm/cm^3)
$C_{tub}(l_s)$ = THO concentration in the perfused fluid (after perfusion) (cpm/cm^3)

In the case of a net flux of water in or out of the tubules and assuming this influx or efflux to be constant with respect to time, the following equation may be applied (Grantham and Burg 1966)

$$P_D = \frac{\O_{tub}(l_o) - \O_{tub}(l_s)}{A} \left[\frac{\ln \frac{C_{tub}(l_o)}{C_{tub}(l_s)}}{\ln \frac{\O_{tub}(l_o)}{\O_{tub}(l_s)}} + 1 \right] \tag{2}$$

where

$\O_{tub}(l_o)$ = volume flow into the perfused tubule (cm^3/cm)
$\O_{tub}(l_s)$ = volume flow out of the perfused tubule (cm^3/sec)
A = area of the perfused nephron (cm^2)
$C_{tub}(l_o)$ = concentration in the perfusion fluid (before perfusion) (cpm/cm^3)
$C_{tub}(l_s)$ = concentration in the perfused fluid (after perfusion) (cpm/cm^3)

In the experiments there was also an influx of potassium into the tubules It is possible to calculate a transport rate coefficient for potassium across the luminal cell membrane into the tubule if certain assumptions are made

1 The transport of potassium occurs only through the tubular cell into the tubular lumen
2 Th

3

furt

T

$$K_1 = \frac{O}{A\ \xi} \ln \frac{C^K_{cell} - \xi\ C^K_{tub}(t_o)}{C^K_{cell} - \xi\ C^K_{tub}(t_s)} \tag{3}$$

where

K_1 = transport rate coefficient for potassium (cm/sec)
O = volume flow through he nephron (cm^3/sec)
A = area (cm^2)
C^K_{cell} = potassium concentration in the tubular cell (mM)
$C^K_{tub}(t_o)$ = potassium concentration in the perfusion fluid (before the perfusion) (mM)
$C^K_{tub}(t_s)$ = potassium concentration in the perfused fluid (after the perfusion) (mM)
ξ = $\frac{C^K{}_{cell}{}^{equil}}{C^K{}_{tub}{}^{equil}}$

All significance calculations were made with the Student t test

Results

The microperfusion rate varied between 14 and 25 nl/min The normal flow rate in the proximal tubules varies between 10 and 35 nl/min (Sonnenberg and Deetje 1964) Some experiments were excluded because of the following reasons

1 If the proximal passage time was longer than 13 sec or the distal appearance time was longer than 45 sec, indicating impaired kidney function (Hierholzer, Wiederholt and Stolte 1966)

2 If the THO activity in the perfused fluid was less than 3 % of that in the perfusion fluid depending on analytical difficulties

3 If large decreases of C^{14} inulin occurred indicating leakage of tubular fluid

TABLE I Calculated mean THO diffusional permeability (P_D) across the proximal tubular wall

Transtubular flux	$\bar{P}_D$ 10^5 cm/sec	n	±1 SD 10^5
zero net flux H_2O	564	18	±241
efflux H_2O	586	10	±262
influx H_2O	230	11	± 65

The results of the diffusional water permeability measurements are presented in Table I The diffusional permeability for water (P_D) was calculated from equation (1) when no net flux occurred and from equation (2) when a net flux occurred At zero net flux of water over the tubular membrane P_D was calculated as 564 10^{-5} cm/sec In the experiments with a small net efflux of water P_D was 586 10^{-5} cm/sec In the case of a net influx of water the value corrected for net flow was 230 10^{-5} cm/sec P_D at zero net flux is significantly higher than the corrected P_D value obtained in experiments with a net water influx ($P<0.001$ but not different from the P_D value determined with a net efflux of water ($P>0.50$) P_D calculated in the experiments with a net efflux of water is however significantly higher than that obtained with a net water influx ($P<0.001$)

The ratios between C^{14} inulin concentration in perfused fluid and C^{14} inulin concentration in the perfusion fluid are calculated in Table II With the use of the isotonic perfusion fluid whose composition was designed to give zero net water flux the inulin concentration decreased on the average by 8 % Morgan Sakai and Ber

TABLE II The ratio (A between the C^{14} inulin concentration in the perfused fluid and in the perfusion fluid

	$\bar{A}$	n	±1 SD
zero net flux H_2O	0 92	18	±0 174
efflux H_2O	0 96	10	±0 139
influx H_2O	0 74	11	±0 098

TABLE III Perfusion of the proximal tubule with the isotonic solution (no 1) Na, K and Cl (mM) and osmolalities (mOsm/kg) in the perfusion and perfused fluids

		$\bar{C}$	n***	±1 SD	P
Na	perfusion fluid	92	12	±7 9	0 50>P>0 10
	perfused fluid*	96	12	±9 9	
K	perfusion fluid	1 3	12	±0 37	P<0 001
	perfused fluid*	3 6**	13	±1 01	
Cl	perfusion fluid	107	6	±5 0	0 05>P>0 02
	perfused fluid*	119	17	±12 7	
Osm	perfusion fluid	326	4	±10 0	0 50>P>0 10
	perfused fluid*	322	11	±8 0	

*=no account is taken of the length of the perfused segment
**=non steady state
***=n refers to all determinations

liner (1968) found a decrease in C^{14} inulin concentration of about 4 % in the rat renal collecting ducts in experiments where they did not expect any net flux This decrease may be due to loss of smaller inulin molecules out of the tubules or to some degree of radiochemical degradation as carbohydrate polymers seem very sensitive to radiation damage (Bayly and Weigel 1960) In experiments with a net efflux the decrease was 4 % With the use of the hypertonic perfusion solution the mean value for the C^{14} inulin ratio decreases by 26 % owing to the relatively high hypertonicity of the solution It must, however, be noted that the intratubular C^{14} inulin concentration (Table II) potassium concentration and osmolality (Table III, IV and V) changes with increasing segment length and therefore these concentration values in different experiments were not strictly comparable

The large net influx of water occurring when using the hypertonic perfusion fluid made it possible to determine the osmotic permeability in a few cases The osmotic water permeability was calculated from the equation given by Ullrich Rumrich and Fuchs (1964) to be 15 10^{-6} cm^3 cm^{-2} sec^{-1} (cm H_2O)$^{-1}$ This value is in good agreement with those found by Ullrich (1966) and Stolte et al (1968)

It is known when using perfusion solutions 1 and 2 that the sodium and chloride concentrations equilibrate within a very short tubular length (unpublished observation) and the intratubular concentrations can therefore within the tubular length used here be regarded as essentially constant

In Table III the changes when the isotonic perfusion fluid was used are shown The results show that there was no increase of the sodium concentration after the perfusion the steady state value for sodium being 96 mM The high potassium concentration in the perfused fluid shows that there was a rapid inflow of potassium into the tubule the dependent

TABLE IV Perfusion of the proximal tubule with slightly hypotonic solution (no 2) Sodium, potassium and chloride concentrations (mM) and osmolalities (mOsm/kg) in the perfusion and perfused fluids

		$\bar{C}$	n***	±1 SD	P
Na	perfusion fluid	100	9	±15 2	P>0 50
	perfused fluid*	94	10	± 7 8	
K	perfusion fluid	0 8	10	± 0 19	P<0 001
	perfused fluid*	3 2**	10	± 0 64	
Cl	perfusion fluid	110	4	± 0 8	P<0 001
	perfused fluid*	120	8	± 2 6	
Osm	perfusion fluid	292	4	± 2 5	P<0 001
	perfused fluid*	315**	8	± 7 4	

*, **, *** see Table III for explanation

fused segment will only affect this finding quantitatively With the assumptions mentioned above a transport rate coefficient (K_1) for potassium influx can be calculated from equation (3) Assuming further, that the intratubular potassium concentration in equilibrium was 4 5 (Marsh, Ullrich and Rumrich 1963) and that the intracellular potassium concentration was 130 mM, a value found in rabbit kidney tubules by Burg and Orloff (1964) the calculated K_1 values was $7\,1 \pm 3\,6 \cdot 10^{5}$ cm/sec (mean value, ±1 SD, n = 10) This K_1 value was calculated with respect to the length of the perfused segment The chloride concentration in the perfusion solution was 107 mM but the concentration in the perfused fluid was significantly higher, with a mean value of 120 mM (P<0 01) As was expected the osmolality was the same before and after the perfusion

Table IV shows the results of perfusion with the hypotonic solution The mean value of sodium concentration in the perfused fluid was not significantly different from the concentration before perfusion (P>0 50) The potassium values showed

TABLE V Perfusion of the proximal tubule with the hypertonic solution (no 3) Sodium, potassium and chloride concentrations (mM) in the perfusion and perfused fluids

		$\bar{C}$	n***	±1 SD	P
Na	perfusion fluid	161	30	±13 1	P<0 001
	perfused fluid*	137**	16	±13 9	
K	perfusion fluid	1 7	38	± 0 60	P<0 001
	perfused fluid*	4 8**	14	± 1 82	
Cl	perfusion fluid	144	12	± 5 8	P<0 001
	perfused fluid*	120	19	±12 4	

*, **, *** See Table III for explanation

a similar trend as in Table III, and the mean value of chloride concentration rose from 110 mM to 120 mM during the perfusion The osmolality increased from a mean value of 292 mOsm/kg to 315 mOsm/kg ($P<0.001$)

Table V shows the results of perfusion with the hypertonic solution During the perfusion the sodium concentration did not reach a steady state concentration as was, however, the case with the chloride concentration, which decreased to 120 mM As in Table III and IV, there was a marked potassium influx during the perfusion

Discussion

THO as an indicator for H_2O

THO as an indicator for water is not quite ideal This is partly because the diffusion coefficient in free water is about 10 % higher for THO than for H_2O at 35° C (Wang, Robinson and Edelman 1953 and Simpson and Carr 1958) Secondly, tritium exchanges with the hydrogen atoms of the hydroxyl and amino groups in the membrane structure The magnitude of such an exchange is unknown for the kidney tubules, but in the special case of a steady state condition with equilibrium between the T atoms in the solution and in the membrane structure this should have no influence on the transport rate of THO Sample collection was therefore not begun until 3—5 min after commencement of microperfusion in order to obtain steady state conditions

Influence of unstirred layers on the diffusional water permeability measurements

The importance of unstirred layers with regard to measurements of diffusional permeability has been carefully investigated by Dainty (1963) The influence of unstirred layers on diffusional water permeability measurements in kidney tubules has been further analyzed by Persson (1969 a) Assuming an unstirred layer equal to cell thickness plus 2 μ on the tubular side of the wall the calculated permeability value is about 25 % smaller than that which would be obtained if no unstirred region existed 25 % probably is an overestimated error since there may well be some stirring effect from the movement of intracellular particles In any case even an under estimation of 25 % does not change essentially the interpretations of the results

In an attempt to diminish the unstirred layer, equation (2) made it possible to measure the diffusional permeability in the presence of either a net water influx or efflux The former creates an inward water flow which would be expected to reduce the P_D value for tritium since its diffusion out of the tubule is thus hindered by a counterstream of water This was in fact observed in the experiments shown in Table I

The interpretation of tritium permeability in the presence of a flow of water moving out of the tubule is however beset with considerable difficulty since the effect of the water stream will be to shift the tritium concentration profile towards and even through the tubular wall, leading to undefined boundary conditions which however, preclude a further analysis However in the experiment the efflux was

apparently insufficient to eliminate the unstirred layer effect The much greater ef fect found in the influx experiment is presumably related to the much higher osmotic driving force for the influx (90 mOsm/kg) compared with that for the efflux (9 mOsm/kg)

Diffusional water permeability The value for diffusional water permeability (wit zero net flux) was 560 10^{-5} cm/sec which is higher than the value of abou 350 10^{-5} cm/sec reported by Peters Baumann and Oelert (1967) It is howeve not possible to comment on this discrepancy

Osmotic water permeability

In water permeability measurements with osmotic gradients L_p the unstirred laye problem does not seem to arise The reason for this is presumably convective move ments at the membrane surface Our value of L_p is 15 10^{-8} cm^3 cm^{-2} sec^{-1} (cm H_2O)$^{-1}$ which is in good agreement with values found by Ullrich Rumrich an Fuchs (1964) Stolte *et al* (1968) and Ullrich (1966)

Difference between osmotic and diffusional water permeabilities

Koefoed Johnsen and Ussing (1953) found that there was a large difference betwee osmotic water permeability (L_p) and diffusional water permeability (P_D) in the frog skin They suggested that the water flow induced by osmotic concentration differ ences was mainly a bulk flow and not a diffusional flow Thau Block and Kedem (1967) studied the movement of water across a series of artificial membranes and concluded that if $g > 2$ 1 ($g = L_p$ $(RT/\bar{V})/P_D$, where $\bar{V}$ is the partial molar volume of water) water transport exists mainly in the form of bulk flow through aq eous pores Cass and Finkelstein (1967) showed that the differences between osmotic and diffusional water permeability found in some investigations on the lipid membranes depended on unstirred layers In experiments with vigorous stirring they found that the diffusional water permeability became as large as the osmotic permeability In lipid membranes all transport is regarded as dependent on diffusion In our experiments we found a ratio g between osmotic water permeability and diffu sional water permeability of 38 This high g value indicates that water is transported across the tubular wall mainly as a bulk flow and that the proximal tubular wall is a highly porous membrane

Water permeability and fluid reabsorption

Ullrich Rumrich and Fuchs (1964) calculated that the L_p value measured is lower than that which would be expected with the water fluxes observed and assuming that the intratubular plasma osmolality differences represent the true driving forces across the tubular wall The measured L_p value would require a transmembrane osmotic difference of 23 mOsm/kg to yield the water flux that exists Such a large osmotic difference has not yet been found It may also be noted that in other organs such as the ciliary body (Auricchio and Barany 1959) and the gallbladder (Diamond

1964), the transcellular osmotic differences are insufficient to explain the normally occurring water flow on the basis of the measured L_p value

In an attempt to explain this and other problems of fluid reabsorption across epithelial membranes, some hypotheses have been put forward One example is the double membrane theory proposed by Curran and MacIntosh (1962) and analyzed thermodynamically by Patlack, Goldstein and Hoffman (1963) Diamond (1964) proposed the existence of local osmosis in the gallbladder, for which Diamond and Tormey (1966) found anatomical support A more extensive discussion about fluid reabsorption has been given elsewhere (Persson 1969 a)

Electrolytes

The electrolyte concentrations were investigated as an extra control in order to exclude large electrolyte movements which would significantly affect the water fluxes

In order to investigate the equilibrium concentrations of electrolytes within the tubules Kashgarian *et al* (1963) injected raffinose solutions into the proximal tubules and removed samples for analysis after sufficient time for equilibration The injected raffinose was reabsorbed to a measurable degree, which means that electrolyte equilibrium was not reached but any a quasi steady state The steady state concentration found for sodium in proximal tubules was 109 mM Extrapolating this value to the equilibrium concentration Gertz (1963) obtained a value of 85 mM These results are in good agreement with our steady state value of about 100 mM for sodium In the experiments where the sodium concentration was initially 161 mM it decreased to a value of about 137 mM, which shows that the contact time and the transport rate for sodium ion was not sufficient to establish a steady state concentration The potassium transport rate coefficient value (K_1 of Eq (3)) must be interpreted with the utmost care Three assumptions (see above) are involved, any of which may not be entirely valid The value of K_1 is high which indicates a fairly rapid influx of potassium into the tubules However, it is also possible using equation (3), if the K_1 value, the ξ value and potassium concentration in the perfusion fluid are known, to calculate the intratubular concentration of potassium in microperfusion experiments if micromethods for potassium determination are not available The chloride concentration in the perfused fluid was always about 120 mM irrespective of which perfusion fluid was used

The results indicate that no major total electrolyte change, which would affect net water movement occurred during the perfusions

Conclusions

In conclusion it can be said that our results are in favour of a bulk flow of water at osmotic gradients across the highly porous tubular wall, whether this is through or between the proximal tubular cells is at present impossible to differentiate The exact mechanism and the driving forces have not been determined conclusively More experimental results are needed for the understanding of the transport mechanism for the fluid reabsorption in the proximal tubules

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Water Permeability in Rat Distal Tubules

By

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Abstract

PERSSON, E *Water permeability in rat distal tubules* Acta physiol scand 1970 78 364—375

Osmotic and diffusional water permeability was studied in rat distal tubules in antidiuresis and water diuresis w
ability was 6 1 ± 1 2
ability 327 ± 132 10
ing the osmotic wate
diffusional water pern
than in antidiuresis
permeability this can only be partly accounted for by the existence of an unstirred layer and is supporting evidence for the concept of aqueous pores, through which the water moves down osmotic gradients mainly as bulk flow and not as a purely diffusional flow The comparatively larger increase in osmotic water permeability than in diffusional water permeability in antidiuresis suggests that ADH can change the pore area pore diameter relation so that the bulk flow increase is larger than the diffusional water flow increase

Knowledge about the water permeability properties in the tubules is of great importance for understanding of how the kidney concentrates the urine Wirtz (1956) showed that the fluid in the first part of the distal tubules was hypotonic Gottschalk (1961) carefully mapped out the osmolarity and its changes along distal rat tubules He showed that in antidiuretic rats the distal tubular urine relatively rapidly became isotonic with plasma On the other hand the osmolarity increase was fairly small in water diuresis (diabetes insipidus) In the dog and in the rhesus monkey however, the distal tubular urine is hypotonic along the entire part of the tubule accessible to micropuncture (Clapp and Robinsson 1966 and Bennet Brenner and Berliner 1968) Ullrich Rumrich and Fuchs (1964) microperfused distal tubules with hypertonic and hypotonic solutions and tried to determine the osmotic water permeability in antidiuresis and in diabetes insipidus The osmotic water permeability in diabetes insipidus must be very low since in perfusions of 1 mm lengths of tubule no net flux of water was detected Their mean value for osmotic water permeability in water diuresis was about one third of the permeability measured in antidiuresis With perfusion solutions containing high calcium concentrations Lassiter *et al* (1965) showed

that calcium decreased the osmotic water permeability in the distal but not in the proximal tubules, which may indicate that calcium is involved in the ADH action mechanism Stolte *et al* (1968) found that the osmotic water permeability increased after adrenalectomy but could be restored to normal values with glucocorticoids The aim of this study was to investigate further the water permeability properties in distal rat tubules by determining the osmotic and diffusional water permeability values in water diuresis and antidiuresis

Methods

Male albino rats of the Sprague Dawley strain weighing between 200 and 300 g were used The rats were fed on standard pellets (Ewos Sodertalje Sweden) The animals were divided [illegible]

glucose solutions Anesthesia was induced by intraperitoneal injection of a 2 1/2 % solution of Inactin® (Chem. Fabrik Promonta GMbH Hamburg) (75—100 mg/kg b w) and maintained with additional small doses The body temperature was regulated with a thermistor thyristor thermostat (Persson, Stahlbrandt and Ulfendahl 1969) The body temperature was recorded from an intrarectal thermistor and the heat was added with a heatable operating table The thermostat works by increasing the effect to the heating element in proportion to the drop in body temperature below 38° A very constant body temperature was achieved. All the rats were tracheostomized in order to assure free air ways Catheters were placed in the right jugular vein and in the left common carotid artery All animals were given 5 ml of infusion sol [illegible]

bladder [illegible]

uoz) In some cases the proximal passage time and distal appearance time were measured after intravenous injection of lissamine green (Steinhausen 1963) Leitz micromanipulators and a Leitz stereomicroscope with a linear magnification of 100 diameter were used The length of the perfused section of the nephron was determined in one of two alternative ways.

1 In those cases where the entire perfused part of the nephron was visible on the surface the length was determined directly with an ocular scale

2 In [illegible] cast [illegible] always made [illegible] lon [illegible] The radius of [illegible]

Three different perfusion solutions having the following compositions were used

1 The sodium concentration of this solution was a modified value of the concentration which Gertz (1963) calculated for the equilibrium value in the distal tubule The weight composition of the solution was NaCl 50 mM KCl 15 mM mannitol 180 mM C^{14} inulin

(33 g/l 100 μCi/ml THO 1 25 mCi/ml Since only a small volume of this solution was prepared the ionic concentrations and osmolality were measured with the same micromethods as used for the perfused samples The following values were obtained Na 56 mM K 16 mM Cl 68 mM, osmolality 320 mOsm/kg It must be noted that the total cation and anion concentrations are not the same

2 The nominal concentration were NaCl 30 mM KCl 10 mM mannitol 50 mM C^{14} inulin (33 g/l 100 μCi/ml) The values obtained at microanalysis Na 33 mM K 10 mM Cl 42 mM and osmolality 140 mOsm/kg

3 The nominal concentrations were NaCl 50 mM, KCl 10 mM mannitol 80 mM C^{14} inulin (33 g/l 100 μCi/ml) The values obtained at microanalysis Na 44 mM K 11 mM [illegible]

Analyses

1 Sodium and potassium were analyzed with an integrating flame photometer (see Persson and Ulfendahl 1969)

2 Chloride was analyzed electrometrically as according to Ramsay, Brown and Croghan (1955) (See Persson and Ulfendahl 1969)

3 Osmolality was analyzed with a microcryometric method as according to Ramsay and Brown (1955) (See Persson and Ulfendahl 1969)

4 THO and C^{14} inulin were analyzed in a Beckman (CPM 200) liquid scintillation counter Sample volumes of between 1 and 10 nl were taken with a constant volume pipette (Danielson Persson and Ulfendahl 1969) A minimum of 6000 counts over background were counted The error of each single determination varied between ±2 5 % ±6 % of the mean value For the statistical analyses see Öbrink (1948) The volume of the scintillation fluid was 10 ml and the composition as follows 485 ml toluene 2 g PPO (2,5 dipenhyloxazole) 0 12 g dimethyl POPOP (1 4 bis 2-4 methyl 5 phenyloxazolyl Benzene) 5 ml ethanol Quenching was constant in all samples

Calculations

The diffusional permeability for THO in a steady state can be calculated as follows The derivation is similar to that given by Sonnenberg Oelert and Bauman (1965) The outward transport of THO J from a small perfectly stirred volume segment ($\pi r^2 \Delta l$) (cm^3) moving forward a distance of dl (cm) in the tubule (which is assumed to have a constant radius) can be determined by

$$J = \frac{dM}{dt} = \frac{d C_{tub}(l)\, \pi r^2 \Delta l}{dt} = \frac{2 \pi r \Delta l \; f_A \; D_{H_2O} \; [C_{tub}(l) - C_p(l)]}{\Delta x} \quad (1)$$

where

M — amount of THO in the segment (cpm)
r = radius of the tubule (cm)
f_A — ratio of total pore area to total membrane area
Δx = thickness of the membrane (cm)
D_{H_2O} = diffusion coefficient for water in the membrane (cm^2/sec)
C_{tub} and $C_p(l)$ = *concentration of THO in the tubule and in the plasma* respectively (cpm/cm^3)
t = time (sec)

The relation between dt and dl is obtained by the equation (2)

$$\text{Ø} \; dt = \pi r^2 dl$$

where Ø is the volume flow of the solution being perfused through the nephron (ml/sec)

Assuming the plasma concentration to be always zero and substituting eqn 2 into eqn 1 yields (3)

$$-\frac{dC_{tub}(l)}{C_{tub}(l)} = \frac{2 \pi r \; f_A \, D_{H_2O} \; dl}{\Delta x \; \text{Ø}}$$

Integrating between the site of the perfusion cannula (l_0) and the site of sampling (l_s) we obtain

$$\ln \frac{C_{tub}(l_0)}{C_{tub}(l_s)} = \frac{2\pi \ r \ f_A \ D_{H_2O} (l_s - l_0)}{\Delta x \ O} \qquad (4)$$

The expression $\frac{f_A \ D_{H_2O}}{\Delta x}$ can now be denoted as the measured permeability of THO, (P_D) (cm/sec) while $2 \pi r \ (l_s - l_0)$ is the total membrane area, A, (cm^2) of the perfused part of the nephron Thus

$$P_D = \frac{O}{A} \ln \frac{C_{tub}(l_0)}{C_{tub}(l_s)} \qquad (5)$$

(See also Grantham and Burg, 1966)

Statistical Calculation

Mean values and standard deviations were calculated by conventional statistical methods All significance determinations were made with the Student t test

Results

The mean blood pressure was higher than 80 mm Hg in all experiments Some experiments were rejected because of the following reasons 1 If the proximal passage time was longer than 13 sec and/or the distal appearance time longer than 45 sec (impaired kidney function) 2 If the THO activity in the perfused fluid was less than 3 % of that in the perfusion fluid (analytical accuracy too low) 3 If large decreases of C^{14} inulin concentration occurred (leakage of tubular fluid)

Diffusional water permeability (P_D)

Table I shows that the diffusional water permeability was about two fold greater in animals in antidiuresis

The experiments were divided into two groups, one in antidiuresis and one in water diuresis The first group consisted of three subgroups

a) normal rats in antidiuresis which excreted urine with an osmolality higher than 310 mOsm/kg

b) hypophysectomized' rats which produced hypertonic urine, indicating that the hypophysectomy had not been successful

c) hypophysectomized rats, which through an i v administration of ADH were excreting hypertonic urine

The second group consisted of animals which produced a urine with an osmolality lower than 310 mOsm/kg after hypophysectomy In the antidiuresis group the dif-

TABLE I Influence of the diuretic state on diffusional water permeability in distal tubules Perfusion with solution (1)

	P_D cm sec	n	± 1 SD cm/sec	P
P_D antidiuresis	327 × 10 [illegible]	25	± 132 × 10 [illegible]	$P < 0\ 001$
P_D water diuresis	157 × 10 [illegible]	21	± 32 × 10 [illegible]	

(33 g/l 100 μCi/ml, THO 1 25 mCi/ml Since onl
prepared the ionic concentrations and osmolalit
as used for the perfused samples The foll
Cl 68 mM, osmolality 320 mOsm/l
centrations are not the same

2 The nominal co
inulin (33 g/l 10
Cl 42 mM and

3 The nominal
inulin (33 g/l, 1
Cl 58 mM, and o

The samples were
under mineral oil T
changes (Hellman U

1 Sodium and potassium
and Ulfendahl 1969)

2 Chloride was analyzed
(1955) (See Persson and U

3 Osmolality was analyzed
Brown (1955) (See Persson

4 THO and C^{14} inulin were
Sample volumes of between 1 a
Persson and Ulfendahl 1969)
error of each single determination
statistical analyses see Öbrink (194
composition as follows 485 ml toluer
1 4 bis 2-4 methyl 5 phenyloxazolyl
samples

Ca

The diffusional permeability for THO in
derivation is similar to that given by Sonne
transport of THO J from a small perfectly s
ward a distance of dl cm in the tubule w
determined by

$$J = \frac{dM}{dt} = \frac{dC_{tub}(l) \cdot \pi r^2 \Delta l}{dt} = \frac{2\pi r \Delta l \; f_A \; D}{}$$

here

amount of THO in the segment (cpm)
radius of the tubule (cm)
ratio of total pore area to total membrane
— thickness of the membrane (cm)
diffusion coefficient for water in the membrane
and C_p l concentration of THO in the tubule
n/cm³
— time sec)

e relation between dt and dl is obtained by the equat

$dt = \pi r^2 dl$

Ø is the volume flow of the solution being perfused th
uming the plasma concentration to be always zero and

$$\frac{C_{tub}(l)}{_{tub}(l)} = \frac{2\pi \; r \; f_A \; D_{H_2O} \; dl}{\Delta x \; \text{Ø}}$$

Integrating between the site of the perfusion cannula (l_o) and the site of samp... we obtain

$$\ln \frac{C_{tub}(l_o)}{C_{tub}(l_s)} = \frac{2\pi \ r \ f_A \ D_{H_2O} (l_s - l_o)}{\Delta x \ \ O}$$

The expression $\frac{f_A \ D_{H_2O}}{\Delta x}$ can now be denoted as the measured permeability (P_D) (cm/sec) while $2\pi r \ (l_s - l_o)$ is the total membrane area, A, (cm², of the perfused segment) of the nephron Thus

$$P_D = \frac{O}{A} \ln \frac{C_{tub}(l_o)}{C_{tub}(l_s)}$$

(See also Grantham and Burg 1966)

Statistical Calculation

Mean values and standard deviations were calculated by conventional statistical methods; significance determinations were made with the Student t test

Results

The mean blood pressure was higher than 80 mm Hg in all experiments Some experiments were rejected because of the following reasons 1 If the proximal transit time was longer than 13 sec and/or the distal appearance time longer than 45 sec (impaired kidney function) 2 If the THO activity in the perfused fluid was less than 3 % of that in the perfusion fluid (analytical accuracy too low) 3 If large decreases of C^{14} inulin concentration occurred (leakage of tubular fluid)

Diffusional water permeability (P_D)

Table I shows that the diffusional water permeability was about two fold greater in animals in antidiuresis

The experiments were divided into two groups, one in antidiuresis and one in water diuresis The first group consisted of three subgroups

a) normal rats in antidiuresis which excreted urine with an osmolality higher than 310 mOsm/kg

b) hypophysectomized rats which produced hypertonic urine, indicating that the hypophysectomy had not been successful

c) hypophysectomized rats which through an i v administration of ADH were excreting hypertonic urine

The second group consisted of animals which produced a urine with an osmolality lower than 310 mOsm/kg after hypophysectomy In the antidiuresis group the dif

TABLE I Influence of the diuretic state on diffusional water permeability in distal tubules Perfusion with solution (1)

	P_D cm/sec	n	± 1 SD cm/sec	P
P_D antidiuresis	327 × 10⁻⁵	25	± 132 × 10⁻⁵	
P_D water diuresis	157 × 10⁻⁵	21	± 32 × 10⁻⁵	$P < 0.001$

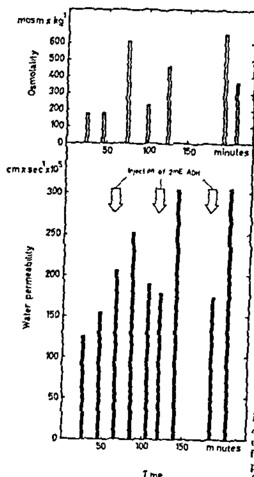


Fig 1 The effect of intravenous injection of ADH on the osmolality of the urine and the diffusional water permeability in a microperfusion experiment on distal tubules of a hypophysectomized rat with diabetes insipidus (mE = milliunit)

fusional water permeability was 327×10^{-5} cm/sec and in the water diuresis group 157×10^{-5} cm/sec The diffusional water permeability was thus about twice as large in the antidiuresis group and the difference was highly significant ($P < 0.001$)

The effect of ADH

The results from one experiment with a rat with diabetes insipidus are shown in Fig 1 Two mU of ADH were injected i v with an immediate transient rise in the arterial blood pressure of about 15 mm of Hg Before the injection the rat produced urine of low osmolality (175 mOsm/kg) and the diffusional water permeability was also low After the ADH injection both the urine osmolality and the permeability rose within 10—15 min before the effect became maximal 30—45 min after the ADH injection the diffusional water permeability and urine osmolality had decreased again to low values The ADH injections were then repeated twice with the same result

TABLE II Ratio (A) between C^{14} inulin concentrations in the perfused and perfusion fluid in all animals Perfusions with solution (I)

	$\bar{A}$	n	± 1 SD
$\frac{C^{14}\text{ inulin concentration in perfused fluid*}}{C^{14}\text{ inulin concentration in the perfusion fluid}}$	0.95	48	± 0.108

* No account is taken of the length of the perfused segment

Net water movement

Table II shows the results from C^{14} inulin determinations where C^{14} inulin was used as a volume indicator These results were obtained from animals in both antidiuresis and water diuresis and were expressed as the ratio between the C^{14} concentration in the perfused fluid and the concentration in the perfusion fluid The mean value of A=0.95 means an increase of 5 % in the volume of the perfused fluid The osmolality also decreased about 5 % (see Table III), which is additional suport for the conclusion of a small net water influx into the tubules However, the osmolality (Table III) and C^{14} inulin concentration (Table II) decrease with increasing segment length and concentration values in different experiments are not strictly comparable

Electrolyte determinations

Table III summarizes the results of the electrolyte determinations It is known that the Na K and Cl (Table III) concentrations equilibrate within a short tubular length (unpublished observations) and therefore with the tubular segment length used here the intratubular concentrations can be regarded as essentially constant No con

TABLE III Mean concentrations (mM) of Na K, and Cl and osmolalities (mOsm/kg) in perfusion fluid and perfused fluids in all animals irrespective of diuretic state Perfusion with solution (I)

		$\bar{C}$	n	± 1 SD	
Na	perfusion fluid	56	27	± 6	P < 0.001
	perfused fluid*	66	34	± 7	
K	perfusion fluid	15.7	23	± 1.3	P < 0.001
	perfused fluid*	12.6	35	± 2.2	
Cl	perfusion fluid	[illegible]	8	± 2.4	P < 0.01
	perfused fluid*	4	31	± 8.0	
Osm	perfusion fluid	320	[illegible]	± 15	0.10 > P > 0.05
	perfused fluid*	304	26	± 20	

sideration is taken of the state of diuresis because the purpose of the table is to show that no major electrolyte changes occurred. The sodium concentration rose significantly from 56 mM to 66 mM while that of potassium decreased significantly from 15.7 mM to 12.6 mM. The chloride concentration rose from 68 mM to 74 mM. It must, however, be noted that the K and Cl concentrations of the perfused fluid did actually differ significantly between the group in antidiuresis and water diuresis. However, the meaning of these differences cannot be discussed since the corresponding plasma ion concentrations are not known.

Osmotic water permeability

The osmotic water permeability L_p was determined with perfusion solutions no. 2 and 3 using C^{14} inulin as the volume indicator. In distal tubules in antidiuresis (solution no. 3) L_p was $6.1 \pm 1.2 \cdot 10^{-8}$ $cm^3 \cdot cm^{-2} \cdot sec^{-1} \cdot (cm\ H_2O)^{-1}$ (mean value ± 1 SD, $n = 8$) and in diabetes insipidus (solution no. 2) L_p was $1.6 \pm 1.6 \cdot 10^{-8}$ $cm^3 \cdot cm^{-2} \cdot sec^{-1} \cdot (cm\ H_2O)^{-1}$ (mean value ± 1 SD, $n = 7$) and in some cases the osmotic flows were too low to measure with any degree of reliability. In some cases 800 µ lengths of tubule were perfused without any detectable volume changes. These results agree with those reported by Ullrich, Rumrich and Fuchs (1964), and the calculations of the osmotic water permeability were made according to them.

Discussion

Criteria for water diuresis and antidiuresis

Measurements of the ability to concentrate urine present certain problems. The negative free water clearance may not be an adequate measure because of the variable amount of urea added to the urine (Thorn 1967). There are also difficulties in obtaining animals with maximal water diuresis. Most anesthetic agents and the performance of a surgical operation are potent releasers of ADH. With the use of Inactin® in normal rats it was difficult to maintain the animals in water diuresis in spite of a large water load (of 5—10 % of the body weight). With ethanol anesthesia and a heavy water load it was possible in some animals to obtain water diuresis even after the operative procedure. To prevent or at least to diminish ADH release hypophysectomy was performed. Unfortunately not even diabetes insipidus resulting from hypophysectomy is a well defined state. After hypophysectomy there may be parts of the hypophysis left which still produce ADH. It is also well known that after some time a new release center is established (Moll and de Wied 1962). According to Thorn (1967) it is probable that even animals with diabetes insipidus produced by electrolytic lesions in the hypothalamic region can release ADH in response to very strong stimuli. Valtin (1966) found in animals with hereditary ADH deficiency that hypertonic urine could be produced by water starvation without the presence of ADH. The reason for this is unclear.

Diabetes insipidus

In this work the experiments were performed in the acute state of diabetes insipidus obtained by hypophysectomy The operation was successful in half of the animals and these animals were used for experiment one to two days after the operation During the first two days postoperatively, large urine volumes with a low osmolality were excreted Subsequently in most animals the urine volumes decreased and the osmolalities increased to 400—500 mOsm/kg After a water load of about 2 % of the body weight the urine production rose and the osmolality decreased to hypotonic values Two problems can be isolated firstly the decreased urinary volume and secondly the increase in osmolality two days after hypophysectomy Engberg Persson and Ulfendahl (1969) measured the glomerular filtration rate in unrestrained and unanesthetized animals before and after hypophysectomy They found a decreased glomerular filtration rate in the animals 1—2 days after hypophysectomy as compared with before which at least in part may explain the decreased urinary volume It may however be noted that Malvin and Fusco (1967) found the glomerular filtration rate not different in rats in antidiuresis and diabetes insipidus Another reason for the decreased urine volume might be an increased aldosterone production It was clearly shown by Hierholzer Widerholt and Stolte (1966) that aldosterone increased sodium reabsorption in both proximal and distal tubules When this diminished urinary volume reaches the collecting duct the flow rate will be low and the contact time presumably long enough for an osmotic equilibration with the renal medulla Gottschalk (1961) showed in hamsters that the renal medulla was slightly hypertonic 400 mOsm/kg in diabetes insipidus

Diffusional water permeability

In the present investigation the experiments were made in the acute state of diabetes insipidus and all animals used produced a hypotonic urine The small scatter of the results seems to indicate that the animals were a homogeneous group even if it was impossible to prove that the animals did not secrete any ADH The group in antidiuresis was not unexpectedly more heterogeneous and the scatter of these results was also larger than in the group with water diuresis It might be argued where the boundary between the two groups should be drawn but a urine osmolality level of 310 mOsm/kg seems to be a fairly reasonable boundary level

The diffusional water permeability (P_D), was 157 10^5 cm/sec in the water diuresis group In antidiuresis the value was significantly higher ($P < 0\,001$) with a value of 327 10^5 cm/sec which is of the same magnitude as that reported by Peters Baumann and Oelert (1967) in antidiuresis

Morel Mylle and Gottschalk (1965) developed a tracer injection technique with THO to determine the water permeability in distal tubules and collecting ducts Though this method is only qualitative it was shown that there is a higher water permeability in antidiuresis than in water diuresis in the distal tubules and the collecting ducts

Osmotic water permeability

The osmotic water permeability (L_p) was calculated according to Ullrich, Rumrich and Fuchs (1964). The nephron was perfused with the hypotonic solutions and the net water outflow was measured. In the original method the volume changes were calculated from the change in osmolality of the perfusion solution (Ullrich, Rumrich and Fuchs 1964). This may be misleading because of diffusion of substances into and out of the tubules and the method seems to give higher values than when C^{14} inulin is used as a volume indicator (Stolte *et al.* 1968). The osmotic water permeability in the present investigation in antidiuresis was $6.1 \pm 1.2 \cdot 10^{-8}$ $cm^3 \cdot cm^{-2} \cdot sec^{-1} \cdot (cm\ H_2O)^{-1}$ (mean value $\pm$ 1 SD, $n = 8$). The value in water diuresis was so low that it was difficult to obtain reliable estimates of the net water fluxes. Therefore the scatter of the results was large and the mean value must be interpreted with the utmost caution. The mean value was $1.6 \pm 1.6 \cdot 10^{-8}$ $cm^3 \cdot cm^{-2} \cdot sec^{-1} \cdot (cm\ H_2O)^{-1}$ (mean value $\pm$ 1 SD, $n = 7$). In some instances in perfusions of 800 μ length of the distal tubules no net fluxes could be detected. These findings are quite in agreement with those of Ullrich, Rumrich and Fuchs (1964), Ullrich (1966) and Stolte *et al.* (1968). Ullrich, Rumrich and Fuchs (1964) found that the mean L_p value in water diuresis was as low as one third of the value in antidiuresis but they claimed that it was probably even lower because of the difficulty of measuring reliably the small water fluxes which occurred.

Difference between osmotic and diffusional water permeability

A relationship between L_p and P_D can be calculated as follows:

$$g = \frac{L_p \cdot \frac{RT}{\overline{V}}}{P_D}$$

where $\overline{V}$ is the partial molar volume of water, and

$$\frac{RT}{\overline{V}}$$

is a correction term to convert L_p into the same dimension as P_D. In the present investigation L_p in water diuresis was about one fourth of the value in antidiuresis. Inserting the actual values found, the g values will be 27 and 15 in antidiuresis and water diuresis, respectively.

These values indicate, according to Koefoed-Johnson and Ussing (1953), that the transport of water at osmotic gradients is mainly a bulk flow and not a diffusional flow. Thau, Block and Kedem (1967) studied the movement of water across a series of artificial membranes and concluded that if $g > 2.1$ the osmotic flow through the membrane is mainly a bulk flow through aqueous pores. If this is correct our results also show that ADH changes the pore area/pore diameter relation so that the bulk flow increase is larger than the diffusional water flow increase.

Dainty (1963) pointed out the important influence of *unstirred layers* on diffu-

sional permeability measurements when the apparent diffusion coefficient in the membrane is approximately as large as the diffusion coefficient in free solution It was shown however (Persson 1969) that unstirred layers will cause only a small error in the diffusional water permeability measurements in the renal tubules In osmotic water permeability measurements in contradistinction to diffusional measurements unstirred layers seem to have no influence on the measurements owing to the existence of bulk flow at the membrane surface

Water structure however may have an important influence on the water transfer through membranes There is some evidence that the water structure in the vicinity of a surface has a more regular structure *i e* is more icelike One of the most sensitive properties with respect to changes in structure is viscosity Derjaguin (1965) found that when water was filtered through finely ground sand the flow was less than expected This was intepreted as indicating an increase in viscosity Fedyakin (1962) studied motion of liquids in microcapillaries and found that the viscosity depended on the capillary radius The significance of these water property changes for flow through pores cannot as yet be evaluated although it seems reasonable to conclude that they must have an important influence

The ADH action mechanism

The mechanism of action of ADH in the kidney has been intensively discussed Grantham and Burg (1966) showed on isolated rabbit tubules that ADH had an effect only on the blood side It seems highly likely that adenosine 3 5′ phosphate is involved in the ADH receptor mechanism (Grantham and Burg 1966 Orloff and Handler 1967) Calcium also seems to interfere in the permeability changes induced by ADH Thorn (1961) showed that the calcium excretion was well correlated to the negative free water clearance High calcium concentrations in the microperfusion solutions diminish the water permeability in rat distal tubules according to Lassiter *et al* (1965) Also pH seems to be of importance (Thorn 1967

Electrolytes

The electrolyte concentrations were investigated as an extra control in order to exclude large electrolyte movements which would significantly affect the water fluxes

In the present investigation with perfusion of isotonic fluid the sodium concentration rose significantly from 56 mM to 66 mM Contrary to this Kashgarian *et al* (1963) found a steady state concentration of sodium of only about 53 mM In the case of potassium it is difficult to determine a steady state concentration This is because the equilibrium concentration rises along the distal tubules and also depends on the metabolic and acid base status of the animal (Giebisch Klose and Malnic 1966) However the mean potassium concentration in the perfused fluid was 12 6 (perfusion fluid concentration 15 7 mM) a finding in good agreement with values reported by Giebisch Klose and Malnic 1966) The results indicate that no major total electrolyte change which would affect net water movement occurred during the perfusions

Water permeability and fluid reabsorption

The varying degrees of fluid reabsorption in the distal tubules are of great importance About 20 % of the filtered volume reaches the distal tubules with an osmolality of about 150 mOsm/kg (Gottschalk 1961) In antidiuresis the urine becomes isotonic before it leaves the distal tubules This osmotic equilibration means a reabsorption of about 10 % of the volume filtered in the glomeruli This very large outflow possibly through pores as a bulk flow, should take solutes along with it In the dog and the rhesus monkey, Clapp and Robinson (1966), and Bennet Brenner and Berliner (1968) did not find osmotic equilibrium in the distal tubules In the dog and rhesus monkey it is probably the collecting ducts which reabsorb the large fraction of water and not the distal tubules as in the rat The low water permeability in the distal tubules of these animals may also explain why they cannot concentrate the urine as much as the rat

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Differentiated Interaction between the Hypothalamic Defence Reaction and Baroreceptor Reflexes

I. Effects on heart rate and regional flow resistance

By

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Abstract

Djojosugito, A M B Folkow, P H Kylstra B Lisander and R S Tuttle Differentiated interaction between the hypothalamic defence reaction and baro receptor reflexes I Effects on heart rate and regional flow resistance Acta physiol scand 1970 78 376—385

As the interrelationship of autonomic patterns involving cortico-hypothalamic and bulbar levels is of general interest the interaction between the hypothalamic defence reaction and the homeostatic baroreceptor reflexes was explored in cats with respect to cardiac as well as vascular effects The results suggest in conformity with Hilton's observations (1963), that defence area stimulation suppresses the baroreceptor reflex inhibition of the heart. On the other hand the baroreceptor influence on vasoconstrictor fibre discharge was largely the same whether defence area stimulations were performed or not This had important haemodynamic consequences especially for muscle blood flow since the reflex inhibition of regional constrictor fibre tone could greatly enhance the cholinergic vasodilatation Therefore such a differentiated interaction between oppositely ... mechanisms has the consequence that the baroreceptor ... act in *synergum* with it with respect to ne... of this are dealt with in the subsequent p...

As various cortico-hypothalamic response patterns of different functional significance (see Folkow Heymans and Neil 1965) can impinge upon the cardiovascular system their interaction with the reflex homeostatic mechanisms has many implications For example the cortico hypothalamic sympatho-inhibitory response may—except when powerfully activated—be effectively buffered by the tonic baroreceptor reflex control as both involve the same efferent links (Lofving 1961) On the other hand, the excitatory defence reaction (see Abrahams Hilton and Zbrozyna 1960)

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acts in direct contrast to the inhibitory baroreceptor reflex, with exception of the effects on flow resistance *in skeletal muscle As the defence reaction by its pressor* effect inevitably activates the baroreceptors the question arises what the net effect of this confrontation between powerful but largely opposite responses will be

Hilton (1963 1965) studied the effects of separate and simultaneous activations of the hypothalamic defence area and the carotid baroreceptors on heart rate and blood pressure in cats He found that the defence reaction suppressed the pronounced bradycardia and depressor response produced by distension of an isolated carotid sinus preparation. This suppression was marked enough to indicate that the defence *reaction completely inhibits the baroreceptor inhibitory reflex, presumably by means* of a central occlusion mechanism It was suggested that such an occlusion might be functionally meaningful since it would allow the all important central influence to produce its full cardiovascular impact in states of emergency where homeostatic mechanisms often have to suffer a temporary abrogation

On the other hand, several earlier studies of vascular control from this laboratory suggest that the baroreceptor reflexes are indeed able to modify considerably the vasoconstrictor fibre activity to the systemic vascular circuits also when these are *simultaneously exposed to the impact of the defence reaction (Feigl Johansson and* Löfving 1962 1964, Folkow, Öberg and Rubinstein 1964) The results of the last mentioned study in the cat even indicated that the baroreceptor inhibition of regional vasoconstrictor fibre tone in the skeletal muscles is a prerequisite for an efficient cholinergic vasodilatation in connection with the defence reaction Such a local interference between these two sets of vasomotor fibres appears to be present in dogs as well though considerably less pronounced in this species (Bolme Ngai and Rosell 1967)

There are thus two series of *results concerning the interaction* between the hypothalamic defence reaction and the baroreceptor reflexes which at first sight appear contradictory It was therefore considered of interest to reexplore this problem in *experiments in* which cardiac and vascular responses were simultaneously recorded and compared It will be seen that the findings in the present and the subsequent study briefly reported elsewhere (Djojosugito *et al* 1968) imply a synthesis of the above mentioned apparently contradictory results with important consequences for the haemodynamic efficiency of the defence reaction

Methods

Experiments were performed on 30 cats of both sexes After induction with ether light anesthesia was maintained by i v administration of chloralose 30—50 mg/kg pentobarbital sodium 30—40 mg/kg or a chloralose urethane mixture 25 and 200 mg/kg respectively A free airway passage was secured by inserting a tracheal cannula

For topical hypothalamic stimulation the head of the animal was fixed in a Horsley Clarke stereotaxic apparatus and a sharp monopolar stainless steel electrode insulated except at the very tip was inserted *into the hypothalamic defence area D A) in 3 kg cats usually cor* responding to the H-C coordinates A 13 to 14 V −2 to −3 and L 1 to 1 5 A Grass model S5 stimulator delivered square wave pulses with a duration of 1—2 msec and an intensity of 2—6 V In later parts of the study a constant current device was used instead, delivering current strengths which were usually set between 0 1—0 5 mA The stimulation frequency was varied between 20—120 imp/sec

Heparin was given as an anticoagulant Blood pressure was measured through a catheter inserted in one of the brachial or femoral arteries, either connected to a mercury manometer writing on a kymograph or, in most cases, to a Statham (P 23 AC) transducer recording on a Grass polygraph When the polygraph was used, heart rate was recorded by means of a tachograph triggered by the arterial pulse waves Muscle blood flow was measured as the outflow from the deep femoral vein, the paw circulation being excluded by a tight ligature at the ankle In some experiments the leg was skinned and the calf muscles isolated so that the venous outflow was derived from this muscle group only After passing a closed optical drop recorder unit the blood was returned to the animal via one of the femoral or external jugular veins When intestinal blood flow was to be measured the abdomen was opened in the mid line The intestines, with exception of a 20—30 g section of the jejunum were extirpated after which the venous outflow from the remaining jejunal section was recorded by another drop recorder unit connected to the superior mesenteric vein Skin blood flow was recorded by the same technique as the venous outflow from the paw by means of a cannula inserted in the great saphenous vein other major collateral veins being ligated Also in these cases blood was returned to the animal via one of the femoral or external jugular veins

In most experiments vascular flow resistance was directly recorded as well The raising speed of the ordinate writer was then determined electronically by the level of the blood pressure, which meant that the number of drops recorded per unit time reflected the flow rate while the height of the ordinates was directly proportional to the blood flow resistance

The vagal nerves were dissected free in the neck and placed on ligatures so that they could be cut during the course of the experiment The central ends were then often arranged for afferent stimulation of the vagal depressor fibres As a rule one of the carotid arteries was gently dissected free and the sinus region was transformed into a blind sac which could be temporarily closed towards the heart by a clamp on the common carotid artery One of the major branches of the external carotid artery was cannulated and the sinus region was connected to a pressure bottle filled with oxygenated dextran Tyrode solution Thus the carotid sinus region could either be exposed to the pulsatile arterial pressure in the normal way or to any desired level of steady pressure by connection to the pressure bottle The sinus nerve on the other side was cut during the course of the experiment

In some experiments the adrenals were exposed transperitoneally and their vessels ligated Adrenocortical substitution was then given as i m injections of hydrocortisone (5 mg/kg Hydrocortal Pharmacia

In most of the experiments the animals were curarized with gallamine iodide (Flaxedil®) 2—4 mg/kg to eliminate any secondary effects on cardiovascular dynamics that could be induced by respiratory changes or by other somatomotor activations in connection with the D A stimulations Constant artificial respiration was then maintained by a respiration pump In order to eliminate the effects of the cholinergic vasodilator fibres atropine 0 3—1 mg/kg b w was given i v during the course of the experiment

Results

Fig 1 illustrates an experiment in which blood pressure heart rate and muscle blood flow resistance were recorded Prior to the recording the vagal nerves and one of the carotid sinus nerves had been cut The remaining intact carotid sinus region was prepared so that it could be exposed either to the systemic arterial pressure via the common carotid artery or be excluded from the systemic circulation by a clamp on the common carotid artery and then exposed to any chosen level of steady pressure from the pressure bottle

In panel A of Fig 1 the defence area (D A) was activated with a standardized submaximal strength while the only remaining carotid baroreceptor (C B) region was exposed to the pulsatile blood pressure This D A stimulation led to an accentuation of the sympathetic drive on the heart which was largely maximal while muscle blood flow increased more than threefold and muscle flow resistance decreased by 50 per cent

In panel B the same D A stimulation was repeated but now the baroreceptors

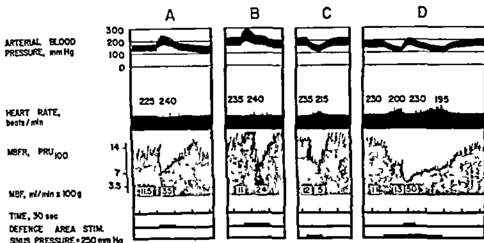


Fig 1 Cat 3 kg flaxedil artificial respiration, vagal nerves and left carotid sinus nerve cut Effects of carotid baroreceptor activation and of standardized, submaximal stimulation of the defence area (D.A), either alone or combined on arterial blood pressure, heart rate, muscle blood flow resistance (M.B F R.) and muscle blood flow (M B F)

A D A stimulation with only one baroreceptor station (right carotid sinus) intact but exposed to the pressure rise caused by the D.A stimulation in the normal manner

B D.A stimulation while the right carotid sinus is excluded from the circulation and instead exposed to a low, constant pressure (about 40 mm Hg)

C Activation of the carotid baroreceptors by exposing them to 250 mm Hg intrasinusoidal pressure

D Same procedure as in C, when the baroreceptor reflex influence is maximal the same D A stimulation as in B is repeated Note the huge increase in muscle blood flow compared with B while almost the same peak level in heart rate is reached

were excluded from the circulation and instead exposed to a low constant pressure, for this reason "resting arterial pressure heart rate and muscle flow resistance were higher Upon D A stimulation the pressure rise was about the same as in panel A and the heart rate reached the same high level The initial increase in muscle blood flow and the fall in muscle flow resistance, were, however now abolished and first after some 20—25 sec a moderate muscle vasodilatation appeared Experiences from other experiments suggest that this delayed muscle vasodilatation was due to adrenaline secretion from the adrenal medulla the initial dilator response brought about by the cholinergic vasodilator fibres during D A stimulation seems to be concealed when the baroreceptor reflex influence is abolished and constrictor fibre tone therefore maintained (compare Folkow, Öberg and Rubinstein 1964)

In panel C a C B stimulation was induced by raising the sinus pressure to 250 mm Hg after clamping the common carotid artery It is seen that this C B stimulation led to a reflex reduction in blood pressure heart rate and muscle flow resistance, while muscle blood flow increased somewhat despite the pressure fall These effects were largely unchanged by atropinization later in the experiment thus reflecting the well known overall inhibition of tonic sympathetic discharge induced by the baroreceptors

In panel D the same C B stimulation was repeated and the same effects as in panel C were obtained Upon this C B stimulation was then *superimposed a D A* stimulation using the same stimulation characteristics as in panels A and B This submaximal D A stimulation led to a prompt rise in heart rate breaking through the reflex bradycardia that was produced by the C B stimulation but the tachycardia did not reach quite the same high level as in panels A and B The pressure rise was about the same as in these panels but due to the lowered initial level the maximal level reached was also lower This was rather characteristic for situations in which a submaximal defence response confronted an intense baroreceptor excitation However now a far more pronounced fall in muscle flow resistance ensued since muscle vasoconstrictor tone was initially inhibited by the continuous C B stimulation This was a characteristic finding in virtually all the experiments Despite a slightly lower perfusion pressure muscle blood flow was now about 50 per cent higher than in panel A where the same D A stimulation was performed but where the baroreceptors were secondarily involved to an extent which must be less powerful than in panel D In other words the intense C B stimulation present in panel D had only a very slight inhibiting influence on the D A effect of the heart despite the fact that the D A stimulation was submaximal On the other hand the C B activation greatly *enhanced* the reduction of muscle blood flow resistance produced by D A stimulation Subsequent atropinization proved that the great increase of muscle flow obtained upon D A stimulation was a consequence of the cholinergic dilator fibres concealed in panel B

Essentially similar results were obtained in all experiments of this type as was also the case when vagal depressor fibres were activated instead (see Fig 2 below) Further when the cardiac parasympathetic fibres of the right vagal nerve were left intact thus allowing for reflex vagal bradycardia upon carotid baroreceptor or vagal depressor fibre stimulations largely the *same* increase in the heart rate was obtained during D A stimulation whether the baroreceptor reflexes were included or not at least when the D A stimulations were fairly powerful On the other hand when very pronounced baroreceptor activations were induced while the D A activations were kept submaximal in strength and in addition reduced with respect to stimulation frequency as well (80 40 and 20 imp/sec respectively) the baroreceptor inhibitory action on the heart tended to overcome the D A stimulatory effect This observation suggests that the D A suppression of the baroreceptor inhibitory action on the heart is not an all or none occlusion phenomenon but rather an expression of a *per se* powerful though in extent graded central suppression On the other hand no real D A suppression of the reflex baroreceptor modulation of the vasoconstrictor fibre discharge was ever seen though very powerful D A activations may for other reasons (see below) completely dominate on the venous side for example

Fig 2 illustrates diagrammatically the effects induced when in addition to blood pressure heart rate and muscle flow intestinal blood flow was recorded as well Here carefully graded stimulations of the depressor fibres in the cut left vagal nerve were used both carotid sinus regions were denervated but the right vagal nerve was

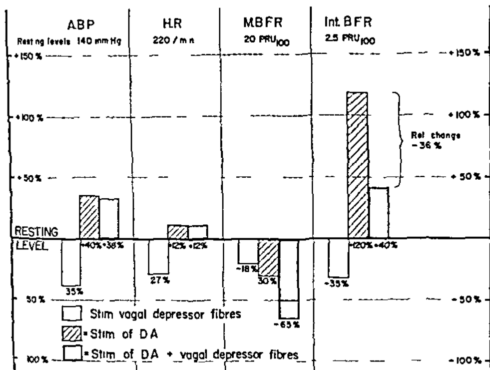


Fig 2 Cat 3 5 kg flaxedil artificial respiration both carotid sinus nerves and left vagal nerve cut Effects of stimulation of vagal depressor fibre and of the defence area (D A) on

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dilatation is greatly *enhanced and the intestinal flow resistance modulated reflexly* to about the same extent as when the depressor fibres were stimulated alone Thus the vasoconstriction induced by the D A stimulation is reduced by 36 % as a result of the depressor fibre activation

initially left intact allowing for reflex vagal bradycardia The results are essentially similar to those shown in Fig 1 Thus D A stimulations completely overpowered the depressor reflex inhibition of the heart rate here also involving vagal bradycardia Further the blood pressure increase upon D A stimulation—which in this experiment *was more powerful than that shown in Fig 1*—was equally extensive whether a simultaneous depressor fibre stimulation was induced or not in conformity with Hilton s observations

On the other hand the depressor reflex considerably enhanced the muscle vasodilatation produced by the standardized D A stimulation and moreover modulated the intestinal flow resistance to largely the same extent (about 35 % reduction), whether the regional vasoconstrictor fibre discharge had been enhanced by D A

In panel D the same C B stimulation was repeated and the same effects as in panel C were obtained Upon this C B stimulation was then superimposed a D A stimulation using the same stimulation characteristics as in panels A and B This submaximal D A stimulation led to a prompt rise in heart rate breaking through the reflex bradycardia that was produced by the C B stimulation but the tachycardia did not reach quite the same high level as in panels A and B The pressure rise was about the same as in these panels but due to the lowered initial level the maximal level reached was also lower This was rather characteristic for situations in which a submaximal defence response confronted an intense baroreceptor excitation However, now a far more pronounced fall in muscle flow resistance ensued since muscle vasoconstrictor tone was initially inhibited by the continuous C B stimulation This was a characteristic finding in virtually all the experiments Despite a slightly lower perfusion pressure muscle blood flow was now about 50 per cent higher than in panel A, where the same D A stimulation was performed but where the baroreceptors were secondarily involved to an extent which must be less powerful than in panel D In other words the intense C B stimulation present in panel D had only a very slight inhibiting influence on the D A effect of the heart despite the fact that the D A stimulation was submaximal On the other hand the C B activation greatly *enhanced* the reduction of muscle blood flow resistance produced by D A stimulation Subsequent atropinization proved that the great increase of muscle flow obtained upon D A stimulation was a consequence of the cholinergic dilator fibres concealed in panel B

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Hilton assumed a more or less generalized suppression of the baroreceptor reflex since the blood pressure increase induced by D.A stimulation was largely the same with or without baroreceptor reflexes in his experiments He therefore proposed that such a general suppression might be of advantage for the optimal cardiovascular impact of the defence reaction in states of emergency However it could not be excluded in Hilton's experiments that the largely unchanged blood pressure rise during baroreceptor involvement might after all be due to a relatively more pronounced cardiac output increase which might then in part result from the preserved baroreceptor adjustment of the systemic vessels In the light of the present experiments such an interpretation of the largely unchanged blood pressure rise seems to be the most likely one For example in many of the present experiments in which the preserved baroreceptor influence on the vessels was obvious (*e g* Fig 2) the pressure rise upon D A stimulation was nevertheless largely the same whether the baroreceptors were activated or not. However in most of the experiments in which the D.A stimulations were kept fairly moderate or even weak the pressure rise was somewhat less prominent when the baroreceptor reflexes were involved despite the usually marked or even complete D A suppression of their impact on the heart No doubt the net balance between the cardiac output increase and the adjustment of the peripheral resistance can vary considerably as this balance depends not only on the pattern of the efferent autonomic discharge but also on the actual state of the cardiovascular effectors haemodynamic factors etc.

Evidently the above mentioned differentiated D.A suppression of the baroreceptor influence on the heart must involve a true nervous occlusion at some *central* level It would otherwise be difficult to explain for example the D.A suppression of the baroreceptor activation of the vagal heart fibres (Fig 2) However concerning the sympathetic cardiovascular control, other types of mechanisms must also be considered when it comes to the final impact on the functionally differentiated cardiovascular compartments *i e* heart resistance vessels and capacitance vessels It is known that D.A stimulation also leads to an activation of the constrictor fibres to the venous capacitance compartment which is highly important for the filling of the heart (*cf* Folkow Mellander and Öberg 1961) It is further known that the frequency response curves concerning the adrenergic fibre control of both the heart (Folkow Lofving and Mellander 1956) and of the capacitance vessels is much more *hyperbolic* than that of the resistance vessels Mellander 1960) Thus the curves for the heart and the capacitance vessels reach nearly maximal levels already at a discharge rate of 4—5 impulses per second levelling off at higher frequencies while for the resistance vessels this is the case first at about 8—12 impulses per second

This circumstance too may have important haemodynamic consequences for the interaction between the defence reaction and the baroreceptor reflexes Suppose that an intense defence reaction—if unopposed by the baroreceptors—produces an increase of the sympathetic discharge rate from a "resting value of 1—2 imp sec to say 6—7 imp/sec Suppose then a baroreceptor activation which curtails this dis-

charge increase to 4—5 imp/sec Because of the abovementioned differences of the frequency response curves this would mean that the effector response would nevertheless be virtually maximal for both the heart and the capacitance compartment while the D A induced resistance increase in many systemic circuits would be substantially less and the muscle blood flow increase further enhanced Such differences in the frequency response characteristics of the various cardiovascular compartments may therefore be of considerable importance in this situation especially for the efficient mobilization of the capacitance vessels for priming the heart apart from the element of central occlusion that seems to be responsible for the intense neurogenic drive on the heart itself

The question arises whether an overall D A suppression of the baroreceptor inhibitory reflexes would have been as haemodynamically beneficial for optimal cardiovascular performance as the differentiated interaction suggested by the present experiments in which only the baroreceptor influence on the heart but not that on the vessels is suppressed It would seem as if such a differentiated interaction would in fact be more efficient from a haemodynamic point of view since—for a given neurogenic drive on the pump—a larger output might ensue This is because the baroreceptor modulation of the resistance vessels would tend to transform part of the pressor load on the left ventricle into an enhanced output to the tissues The final aim of the cardiovascular system is after all to establish nutritional flow and certainly so also in emergency situations and the results suggest that the muscles are especially favoured in this respect

A differentiated interaction between the defence reaction and the baroreceptors may be especially important in all those species which appear to have few or perhaps no cholinergic vasodilator fibres in the muscles Uvnäs findings (1967) suggest that only or mainly carnivores have a rich supply of such fibres Other species would have to rely upon an inhibition of vasoconstrictor fibre tone in the skeletal muscles and the delayed (some 20—25 seconds in cat) vasodilator action of bloodborne adrenaline If parallels may be drawn to the cat a constrictor fibre inhibition alone would reduce muscle flow resistance from some 20 PRU_{100} to perhaps 7—10 PRU_{100} which—with a pressure rise from 100 to 130—140 mm Hg—would increase muscle blood flow from some 5 ml/min × 100 g to 15—20 ml/min × 100 g If so the excitatory defence reaction and the baroreceptor inhibitory reflexes being in most respects each others contrasts would act in synergism when simultaneously induced because of the selective suppression of the baroreceptor impact on the heart The effects with respect to cardiac output and work load on the heart are dealt with in the subsequent paper (Kylstra and Lisander 1969) It seems likely that a similar differentiated interaction between the central autonomic drive and the homeostatic baroreceptor reflexes might occur also during *e g* intense exercise

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Differentiated Interaction between the Hypothalamic Defence Area and Baroreceptor Reflexes

II. Effects on aortic blood flow as related to work load on the left ventricle

By

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Abstract

Kylstra P H and B Lisander *Interaction between the hypothalamic defence area and baroreceptor reflexes II Effects on aortic blood flow as related to work load on the left ventricle* Acta physiol scand 1970 78 386—392

In experiments on cats the interaction between the oppositely directed hypothalamic defence reaction and the baroreceptor reflexes was analysed with particular regard to the effects on aortic blood flow left ventricular work load and muscle blood flow Because of their differentiated interaction — *suppression of the baroreceptor reflex effect* on the heart with preservation of the reflex modulation of the vascular bed (Djojosugito *et al* 1969) — the baroreceptor reflex so modifies the primary defence reaction, with its intense neurogenic drive on the heart, that a greater cardiac output is gained for a given left ventricular work load This particularly favours muscle blood supply It follows that such a differentiated interaction between two basically opposite autonomic patterns causes them to act in synergism with respect to efficient cardiovascular performance in states of emergency

In the previous study (Djojosugito *et al* 1969) evidence was presented to show that activation of the hypothalamic defence area (D A) markedly suppresses the baroreceptor reflex inhibition of the heart, in agreement with earlier findings by Hilton (1963 1965) On the other hand the baroreceptor reflex modulation of the systemic resistance vessels proved to be about as potent in producing reductions of constrictor fibre discharge whether the defence area was stimulated or not In the present study the net result of this differentiated interaction between the excitatory defence reaction and the reflex inhibitory response was explored with particular respect to the effect on aortic blood flow as related to the neurogenic drive and total work load of the left ventricle Part of the present results have been reported briefly in a preliminary communication (Djojosugito *et al* 1969)

Methods

35 cats were used ranging in weight from 2 5—4 3 kg After induction with ether the animals were anesthetized lightly with i v administration of either chloralose, 30—50 mg/kg or

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pentobarbital sodium, 30—40 mg/kg A free airway passage was secured by means of a tracheal cannula

For topical stimulation of the hypothalamic defence area (D ... echnique was ... ave pulses of ... ted to a con ... aried direct- ... imp/sec.

... rom here on, for ... was recorded by an electromagnetic flowmeter, Medicon 2000 For this purpose the sternum was divided in the midline with a rotating saw and the thorax opened A well fitting probe was placed around the root of the aorta after which the chest was again closed and the animal kept on positive pressure artificial respiration The product of mean arterial blood pressure and aortic blood flow was used as a rough estimate of left ventricular work To reveal the primary autonomic cardiovascular adjustments the animals were 'curarized' with gallamine triethiode (Flaxedil, May & Baker, 2—4 mg/kg b w), thus eliminating respiratory changes or other somatomotor activity that may be induced by the defence area stimulations

In one of the animals (3 5 kg) the electromagnetic probe was placed around the aorta under pentobarbital anesthesia and under aseptic precautions one week before the acute experiment. In this way it was possible to compare aortic flow in the awake animal with that during anesthesia. Further, the fairly traumatic thoracic preparation could be avoided during the course of the acute experiment

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... a Statham (P 23 AC) strain gauge pressure ... recording on a Grass polygraph Heart rate was recorded by means of a tachograph triggered by the arterial pulse waves Muscle blood flow was recorded in all the experiments and measured as the outflow from the popliteal vein by means of a closed drop recorder unit operating an ordinate writer The circulation to the paw was always eliminated by a tight ligature placed around the ankle, sometimes the calf muscles were skinned as well to eliminate any admixture of cutaneous blood flow Muscle blood flow resistance was directly recorded as described in the previous paper (Djojosugito *et al* 1969)

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on one side was crushed w ...
visible branches cranial to th ... ligated so that a blind sac was obtained, which could be intermittently closed towards the heart by a clamp on the common carotid artery Via a cannula in one of the major branches of the external ...
baroreceptor ...
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nection to ...
barorecepto ... the pulsatile systemic blood pressure In this way the arterial pressure rise produced by the defence area stimulation could activate the baroreceptors or they could either be kept largely unstimulated by keeping the sinus pressure at about 40 mm Hg or suddenly be activated at high distending pressures from the pressure bottle

Atropine (0 2—1 mg/kg b w) was given in the course of the experiments to test to what extent the vasodilatation produced by D A stimulations was of cholinergic origin This was, however, also clear from the fact that the fall in muscle flow resistance was usually far beyond that seen upon elimination of constrictor fibre tone

Results

The present results are best summarized by reference to Fig 1 showing the mean values of 8 technically successful experiments, in which the same measurements were performed throughout *i e* of mean arterial blood pressure (A B P), heart rate

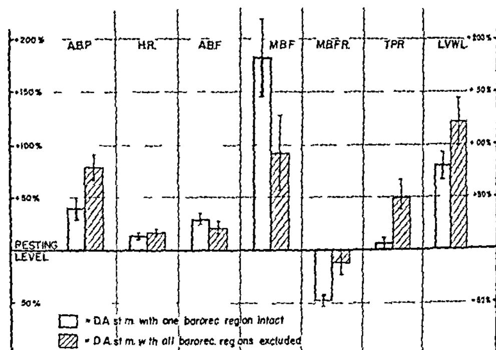


Fig 1 Compiled results from 8 experiments of ... hypothalamic defence area Effects on arterial blood pressure ... flow (A B F) muscle blood flow (M B F) ... peripheral resistance (T P R) and calculated left ventricular work load ...
The grey columns represent defence area stimulations under conditions in which only one of the baroreceptor regions is left active The crosshatched columns represent the effects of identical defence area stimulations when all baroreceptor reflex modulations are excluded.

(H R) aortic blood flow (A B F) and muscle blood flow (M B F) and from which muscle blood flow resistance (M B F R) total peripheral resistance (T P R) and left ventricular work load (L V W L) were calculated These measurements were performed first during submaximal D A stimulations while the secondary involvement of the baroreceptor reflexes was totally excluded both vagal nerves and left carotid sinus nerve being cut and the isolated right carotid sinus exposed to a constant low transmural pressure Then the same D A stimulations were repeated while the previously isolated right carotid sinus region was now exposed to the arterial blood pressure via the common carotid artery so that the corresponding baroreceptors were activated in the normal way by the pressure rise caused by the D A stimulation The mean values in Fig 1 show that even the involvement of only one of the baroreceptor stations was enough to modify markedly most of the effects of the D.A. stimulation The only clear exception was the neurogenic heart rate increase which was largely the same whether the baroreceptor reflex was excluded or not Thus muscle blood flow resistance was much more reduced when the baroreceptor reflex was involved Aortic flow was significantly more increased while mean arterial pressure rise was considerably less pronounced as was the increase of left ventricular

work load (L V W L) and the rise in the total peripheral resistance (T P R) In other words, for one and the same neurogenic drive on the heart more cardiac out put was gained at a relatively lower work load for the left ventricle because of the baroreceptor modulation of the systemic blood vessels In addition the blood supply to the muscles was especially favoured

As the experiments necessitated rather extensive interference with the animals involving an opened thorax and positive pressure breathing it is obvious that the induced neurogenic adjustments of overall haemodynamics were not identically balanced in all the animals However, all the neurogenic effector responses (H R M B F M B F.R and T P R.) exhibited principally the same direction of change in practically all the experiments though such factors as A B P being the result of the *ratio* between cardiac output and T P R and L V W L being the *product* of A B F and A B P could vary considerably

Therefore in most of the present experiments the involvement of the baroreceptor reflex meant a substantial reduction of the arterial pressure rise that was produced by the submaximal D A stimulation This implies that the gain in A B F increase was usually not quite as marked as to balance the baroreceptor reflex reduction of the T P R rise produced by the D A stimulation Such an experiment is shown in Fig 2 Panel A of this figure shows the increase of A B F A B P calculated L V W L H R M B F and the decreased M B F R produced by a submaximal D A stimulation when the only remaining baroreceptor station the right carotid sinus was exposed in the normal way to the arterial blood pressure changes — In panel B the carotid sinus was excluded from the circulation and instead exposed to a low constant pressure The increase of A B F was now somewhat smaller but the rise in A B P markedly higher so that L V W L was nevertheless decidedly larger At the same time the decrease of M B F R was now minimal though the pressure rise led to a flow increase — In panel C the carotid sinus was first exposed to 250 mm Hg pressure which reflexly lowered the A B P H R and M B F R with only a slight decrease in A B F as a result While this baroreceptor activation was still going on the same D A stimulation as in panels A and B was started A B F now increased to nearly the same level as in panel B but L V W L was only about half as large Because the baroreceptor inhibition of the sympathetic discharge to the heart was here very intense and the D A stimulation was decidedly submaximal in strength the D A break through with respect to heart rate was incomplete the reasons for which are discussed in the previous paper (Djojosugito *et al* 1969) Nevertheless the heart delivered nearly the same output as in panel B especially favouring the skeletal muscles but at a far lower metabolic cost as judged from the relative changes in L V W L

Fig 3 illustrates another experiment in which the overall haemodynamic balance is somewhat different during the interaction between the defence reaction and the baroreceptor reflex despite the fact that the neurogenic effects on heart and blood vessels are principally the same as in Fig 2 In panel A the carotid baroreceptors were isolated from the circulation and suddenly exposed to a steady pressure of 200

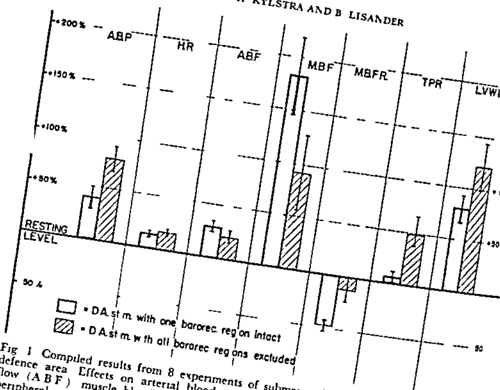


Fig 1 Compiled results from 8 experiments of submaximal stimulations of the hypothalamic defence area. Effects on arterial blood pressure (A B P), heart rate (H R), aortic blood flow (A B F), muscle blood flow (M B F), muscle blood flow resistance (M B F R), total peripheral resistance (T P R) and calculated left ventricular work load (L V W L). The grey columns represent defence area stimulations under conditions in which only one of the baroreceptor regions is left active. The crosshatched columns represent the effects of identical defence area stimulations when all baroreceptor reflex modulations are excluded

(H R), aortic blood flow (A B F) and muscle blood flow (M B F) and from w… muscle blood flow resistance (M B F R), total peripheral resistance (T P R) … left ventricular work load (L V W L) were calculated. These measurements w… performed first during submaximal D A stimulations while the secondary invol… ment of the baroreceptor reflexes was totally excluded, both vagal nerves and le… carotid sinus nerve being cut and the isolated right carotid sinus exposed to a con… stant low transmural pressure. Then the same D A stimulations were repeated while the previously isolated right carotid sinus region was now exposed to the arterial blood pressure via the common carotid artery, so that the corresponding baroreceptors were activated in the normal way by the pressure rise caused by the D A stimula… tion. The mean values in Fig 1 show that even the involvement of only one of th… baroreceptor stations was enough to modify markedly most of the effects of the D A stimulation. The only clear exception was the neurogenic heart rate increase which was largely the same whether the baroreceptor reflex was excluded or not. Thus muscle blood flow resistance was much more reduced when the baroreceptor reflex was involved. Aortic flow was significantly more increased while mean arterial pres… sure rise was considerably less pronounced, as was the increase of left ventricul…

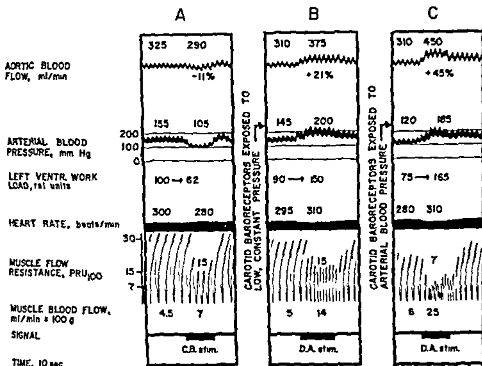


Fig 3 Cat, 3 kg, chloralose 50 mg/kg b w Vagotomized, left sinus nerve cut, artificial respiration, curarized with Flaxedil Same measurements as in Fig 2 Panel A Effects of exposing the right carotid sinus region to a pressure of 250 mm Hg Panel B Effects of defence area stimulation when the baroreceptor reflex modulation is excluded by exposing the carotid sinus to a low, constant pressure Panel C Effects of an identical defence area stimulation when the carotid baroreceptors are exposed to the arterial blood pressure and therefore reflexly involved by the pressure rise

appreciably increasing the total work load for the left ventricle, because the level of ABP reached, was not quite as high This experiment, and that shown in Fig 2 of the preceding paper (Djojosugito *et al* 1969), presumably reflects the haemodynamic balance obtained in Hilton's experiments (1963 1965)

It is clear that a great number of factors can effect the balance between the complex neurogenic adjustments of heart blood flow resistance and the venous capacitance seen in these situations Therefore it is not surprising that the net ef fects on cardiac output arterial blood pressure and work load on the heart can vary considerably, as is illustrated by these two experiments, even though the basic neurogenic interaction between the defence reaction and the baroreceptor reflexes is principally the same In any case the results strongly suggest that the involvement of the baroreceptor reflexes will so modify the primary defence reaction as to produce an enhanced cardiac output increase for one and the same neurogenic drive on the heart and work load for the left ventricle This enhanced output increase favours especially the muscle vascular bed

Discussion

In close agreement with the results of the preceding paper (Djojosugito *et al* 1969) the present experiments suggest that the interaction betwen the hypothalamic defence area and the baroreceptor reflex is a differentiated one Thus, the baroreceptor reflex influence on the heart is centrally suppressed by the defence reaction in agreement with Hilton's finding (1963, 1965), while the baroreceptor reflex modulation of the vascular bed remains largely unaffected by a simultaneous activation of the defence area

Further, the results show that this has important consequences, not only for the heart itself, but also for the tissue nutrition, as the neurogenic drive on the pump becomes so 'transformed' as to yield more of an output increase for a given total work load on the myocardium In fact, in the majority of the present cases in which the defence area activations were submaximal in extent, an enhanced CO increase was gained at a reduced arterial pressure increase often to such an extent that it implied a relatively reduced work load for the left ventricle Further the increased output tended to favour especially the blood supply of the skeletal muscles even though the rise in perfusion pressure was in most cases less prominent when the baroreceptors were involved

Since the primary purpose of the cardiovascular system is to provide flow not pressure and during defence reactions presumably to provide nutritional flow especially to the skeletal muscles the maintenance of the baroreceptor adjustments of the vascular bed as combined with the suppression of their cardiac effects must be considered as being of great advantage in fact it supports the defence reaction rather than counteracts it In other words, two neurogenic adjustments which on the whole are opposite in direction—the excitatory defence reaction and the inhibitory baroreceptor reflex—become in essence *synergistic* in terms of the haemodynamic results because of such a differentiated interaction

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The Effect of Epinephrine and Dibutyryl 3',5' Cyclic AMP on the Incorporation of ^{32}P Inorganic Phosphate into Adenine Nucleotides and Guanine Nucleotides of the Intact Isolated Rat Diaphragm

By

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Abstract

Walaas, E and O Walaas *The effect of epinephrine and dibutyryl 3',5' cyclic AMP on the incorporation of ^{32}P inorganic phosphate into adenine nucleotides and guanine nucleotides of the isolated intact rat diaphragm* Acta physiol scand 1970 78 393—403

The influence of epinephrine and dibutyryl 3',5' cyclic AMP on the incorporation of ^{32}P inorganic phosphate [illegible] butyryl 3',5' cyclic AMP on the specific activity of intracellular inorganic phosphate was observed It is indicated that the increased incorporation of ^{32}P inorganic phosphate into mononucleotides promoted by epinephrine is due to activation of adenyl cyclase as well as to increased influx of ^{32}P inorganic phosphate from the medium

Key words Epinephrine 3 5 cyclic AMP, P^{32} labelling, ATP GTP Ouabain, Inorganic P

Many metabolic effects of epinephrine on muscle can be explained by a mechanism involving activation of the adenyl cyclase system (Sutherland *et al* 1965), which gives rise to increased formation of cyclic 3' 5' AMP This hormonal 'messenger' is known to activate phosphorylase kinase and glycogen synthetase kinase Cyclic 3',5'

Abbreviations ATP, ADP AMP Adenosine tri-di mono-phosphate Dibutyryl 3 ,5 cyclic AMP N^6,2-0-dibutyryl adenosine 3 5 cyclophosphate GTP GDP GMP Guanosine tri di mono-phosphate

AMP acts moreover as an activator of other reactions in muscle such as the nephrine induced lipolytic response in the rat heart (Challoner and Steinberg 19 Evidence has also been given that cyclic 3′5′ AMP is a mediator of the ionotr response in the working heart to epinephrine (Øye *et al* 1964) and the relaxing tion of epinephrine on smooth muscle mediated by *a* receptors (Andersson Mohme Lundholm 1968) These observations have led to the concept that epin rine due to formation of cyclic 3′,5′ AMP promotes increased utilization of AT several independent reactions

In the present study the effect of epinephrine on the incorporation of ^{32}P inorg phosphate into adenine nucleotides and guanine nucleotides in the rat diaphragm been investigated It has been shown that when the intact diaphragm is incub in the absence of added substrate epinephrine promotes increased incorporatio ^{32}P into ATP and ADP as well as into GTP and GDP Similar effects have b observed when the diaphragm is incubated in the presence of dibutyryl 3′5′ cy AMP In further experiments the effect of epinephrine on the turn over of ^{32}P mononucleotides has been investigated in relation to the ionic composition of medium and during inhibition of ion membrane transport by ouabain

Experimental procedure

Incubation technique Female rats weighing 200—250 g from a local strain (original W s were used The animals were fasted for 18 hours and killed by a blow on the head and capitated The diaphragms were dissected out chilled in ice cold medium and the so ca intact preparation according to Kipnis and Cori (1957) was prepared Before incubation preparation was divided into two halves according to Kono and Colowick (1961) One was used as control the other half incubated with addition of hormone Two or three h diaphragms together were incubated in 100 ml Erlenmeyer flasks Soaking of the diaph at 37

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Thereupon the preparations were transferred to chilled media and the diaphragms rap dissected out and frozen in liquid nitrogen For the determination of intracellular inorga phosphate the technique was slightly different the diaphragms being rapidly transferred to chilled plate blotted frozen and weighed

Extraction procedure The frozen diaphragms were weighed on a torsion balance a mechanically powdered in a stainless steel mortar cooled with liquid nitrogen The fro powder from two or three hemidiaphragms weighing 600—900 mg was homogenized in 5 10 % perchloric acid (PCA) for 5 min in an all glass Potter Elvehjem homogenizer cooled an ice bath The homogenate was centrifuged for 10 min at 10 000 × g in a Sorvall SS cent fuge at +2° C The residue was reextracted twice with 2 ml 10 % perchloric acid and [illegible] ocedure

the acid soluble nucleotides we

Tsuboi and Price (1959) Ther

atography (TLC) according

following 10—20 mg Nuchar

sk The Nuchar C had been pr

viously washed two times with 0 1 N HCl and three times with distilled water A stock solution of Nuchar C containing 10 g in 100 ml H_2O was made After dilution of the stock solution with H_2O (1 6) 2 ml was added to the PCA extract After shaking for 15—30 min in the cold ... otides as well as pyrimidine nucleotides onto Nuchar ... ticles and the adjustment of pH for proper elution ... (1963), except for the use of phosphate buffer In ... ere preferred to remove perchloric acid and per ... final washing with 0 05 M acetate buffer, pH 4 5 ... Nuchar C was achieved by shaking with 6 ml 2 5 % pyridine in 50 % ethanol in Erlenmeyer flasks for 2 hrs at 37° C in a water bath Nuchar C was removed by centrifugation and filtration through a sintered glass filter The eluting solvents were evaporated in vacuo over night

Thin layer chromatography The residues were dissolved in 170 μl H_2O and appropriate volumes were applied on polyethyleneimine cellulose plates Two dimensional thin layer chromatography (LiCl at three concentrations 0 3 M, 0 8 M and 2 0 M in the first dimension and sodium formate at four different concentrations 0 25 M, 1 0 M, 2 0 M and 4 0 M in the second ... nderath (1964) The ... UV absorption with ... England) Guanine ... light after exposing the plates to conc ... vapour for 5 min The powder from each spot was removed with a small spatula trans ferred to alumina plates and counted in a windowless methane flow counter (Frieseke and Hoepfner)

Pho... were ... tem ... at I ... ml H_2O_2 Phosphorus was determined by the method of Fiske and Subbarow (1925) Powder "blank spots" from each plate were extracted and treated in the same way as the nucleotides spots Phosphorus content of the "blank spots" did not exceed 0 01 μg and was found to be rather constant The results are expressed as cpm/μg P of nucleotides (specific activity) or as percentage of control ... of inorganic ... and Chat ... organic p ... perchloric ... ugation phosphorus was determined on aliquots of the supernatant by the same method

Phosphorylase was measured by the method of Cornblath *et al* (1963

Recovery of nucleotides A near quantitative recovery (85 %—95 %) of ATP UTP and CTP was obtained when these compounds were added as internal standards to PCA extracts and carried through the whole procedure of TLC However recovery of the ... (GTP, GDP and GMP) added as "internal standard ... the procedure was only 50 %—60 % The recovery ... jected to TLC without charcoal treatment was 92 % ... attributed to the ... onto charcoal ... Nuchar C was ... recovery was f ... of 47 % when guanine ... guanine nucleotides pr ... tion procedure It has ... a considerable breakdown of nucleoside ... of the nucleoside diphosphates observed ... phosphorylation of the nucleoside triphc ... is true for the relatively large amounts of IMP observed

Calculation of specific radioactivities

The specific ac ... ion that the β phosphate o ... This is in accordance wi ... (1961) and Clauser *et al* ... incubation period used in the present study the specific activity of the terminal phosphate in ADP was ... wer than that of the γ phosphate in ATP Th ... not take part in the adenylate kinase reactio ... terminal phosphate in GDP and the γ pho ... The labelling of the α phosphate in

TABLE I Acid soluble adenine nucleotides and guanine nucleotides in the intact rat diaphragm after incubation for 10 min without substrate in the absence and the presence of epinephrine ± Standard error of the mean P Probability of the significance of the difference of controls versus epinephrine ( ) Number of experiments

Group	ATP (μmoles per g wet tissue)	ADP	5'-AMP	GTP (μmoles per g wet tissue)	GDP	5 GMP
Control (6)	2 86* ±0 20	0 66 ±0 07	0 13 ±0 033	0 094* ±0 012	0 070 ±0 025	0 152 ±0 031
Epinephrine (1 μg/ml) (6)	2 34* ±0 15	0 60 ±0 06	0 10 ±0 025	0 064* ±0 006	0 064 ±0 009	0 13? ±0 032

* P 0 02

AMP and GMP was very low and no correc has been made in the calculation of specific

As will be presented below the uptake of intact diaphragm is rather slow However content of inorganic phosphate in the mediu specific activity decreased by 20—30 per cent This can be attributed to a rapid exchange of phosphate between the medium and the damaged muscle fibres of the tissue preparation along the rib cage The large and somewhat variable changes in the phosphate content and in the specific activity of the medium complicate the determination of the specific activity of intra cellular inorganic phosphate The phosphate content and the specific activity of the medium at the end of the incubation period were used as an expression for these figures in the extra cellular space of the diaphragm An extracellular space of 18 per cent in the intact diaphragm was used in the calculations (Kipnis and Cori 1957)

Materials

Adrenaline bitartrate (Rhc mg epinephrine dissolved u and 10—50 μl added to 5i

Radioactive phosphate (Na₂H³²PO₄) at a concentratio Institutt for Atomenergi Kjeller Norway 400 μC of the neutralized solution was added to

and nucleotide standards were ob U S A

C Vegetable Carbon from Chemical Division West Virginia Pulp and Paper Co New York The material for thin layer chromatography was *Cellulose* powder MN 300 from Macherey Nagel & Co Germany and *Polyethyleneimine* (PEI) from Badische Anilin and Sodafabrik Germany

Results

The influence of epinephrine on the content of adenine nucleotides and guanine nucleotides in the diaphragm is shown in Table I It is seen that after a short incubation time of 10 min the content of ATP and GTP were both slightly decreased by epinephrine This observation may be due to an activation of adenyl cyclase by

TABLE II The effect of epinephrine on the incorporation of ^{32}P inorganic phosphate into adenine nucleotides and guanine nucleotides of intact rat diaphragm incubated in Krebs Ringer bicarbonate medium for 10 min in the absence of substrate Experiments performed in the presence of 1 mM caffeine are also presented
( ) Number of experiments

Group	ATP*	ADP**	AMP	GTP*	GDP**	GMP
	Specific activity (cpm/μgP)					
Control (4)	734±40	404±26	27±7	944±82	970±90	94±19
Epinephrine 1 μg/ml (4)	1374±117	541±32	18±8	1361±123	1216±194	94±28
P	0 001	0 005	<0 10	0 05	0 10	<0 10
Caffeine (4)	782±40	364±41		922±186		
Epinephrine (0 2 μg/ml + Caffeine (4)	1510±58	540±73		1422±152		
P	0 001	0 10		0 05		

* Refers to γ P of ATP and GTP
** Refers to terminal P of ADP and GDP

epinephrine with increased production of cyclic 3′,5′ AMP which by stimulation of kinase reactions result in some dephosphorylation of nucleoside triphosphates

The effect of epinephrine on the incorporation of ^{32}P inorganic phosphate into nucleotides is presented in Table II The specific activity of the γ phosphate group of ATP was almost doubled and the specific activity of the γ phosphate group of GTP and the terminal phosphate group of GDP were increased to the same level The specific activity of the terminal phosphate group of ADP was lower than the γ phosphate of ATP in the controls and only to a smaller extent increased by epinephrine The reported effects were obtained by a concentration of 1 0 μg/ml of epinephrine, but the effects of 0 2 μg/ml of epinephrine (not included) were of the same order of magnitude In the presence of caffeine which is known to increase the level of cyclic 3′ 5′ AMP by inhibition of phosphodiesterase (Sutherland and Rall 1960) the epinephrine effect on ^{32}P turnover in ATP and GTP was slightly enhanced (mean values) although not at a significant level

The experiments reported in Table III indicate the possibility that the epinephrine effect on the nucleotides is related to an effect on the specific activity of intracellular inorganic phosphate It is seen that the content of intracellular inorganic phosphate was slightly decreased by epinephrine while the specific activity was doubled during incubation for 30 minutes The penetration of ^{32}P inorganic phosphate into diaphragm is slow The specific activity of intracellular inorganic phosphate was increased by epinephrine from 1 3 per cent to 2 9 per cent of the specific activity of

TABLE III *The influence of epinephrine on the incorporation of ^{32}P inorganic phosphate int intracellular inorganic phosphate Intact rat diaphragm incubated in Krebs Ringe bicarbonate medium without substrate for 30 min*
( ) Number of experiments

Group	Intracellular inorganic P		
	mg P/g wet tissue	Specific activity cpm/µgP	Relative specific activity (% Extracellular P)
Control (8)	*0 38±0 028*	*3290±450*	*1 3±0 2*
Epinephrine (1 µg/ml) (8)	0 32±0 032	6840±725	2 9±0 3
P	0 10	>0 001	>0 001

TABLE IV *The effect of dibutyryl 3 , 5 cyclic AMP on the incorporation of ^{32}P inorganic phospha into intracellular inorganic phosphate, ATP and ADP Incubation of intact rat dia phragm in Krebs Ringer bicarbonate medium containing 1 mM caffeine but withou addition of substrate 15 min soaking period followed by 30 min incubation at 37 C*
( ) Number of experiments

Group	Intracellular inorganic P***		ATP* cpm/µg P	ADP**
	cpm/µg P	Relative specific activity (% extracellular P)		
Control (8)	3365±295	1 4±0 3	2305±200	1090±6[illegible]
0 5 mM *Dibutyryl* 3 5 cyclic AMP	3060±320	1 3±0 3	3290±375	1310±36
P	<0 10	<0 10	>0 01	>0 01

* *Refers to γ P of ATP*
** *Refers to terminal P of ADP*
*** *Determination of specific activity of inorganic P and ATP was done in separate experiments There was a considerable variation of specific activity of ATP and inorganic P in different experiments*

inorganic phosphate in the medium during 30 min incubation It has previously been shown that the specific activity of γ P of ATP as well as of intracellular inorganic phosphate increases lineary with the incubation time (Walaas *et al* 1969) Thus it appears that the specific activity of ATP (calculated for an incubation time of 30 min) is lower than the specific activity of the intracellular inorganic phosphate both in the absence and in the presence of epinephrine

TABLE V The effect of epinephrine in the presence of 0 2 mM ouabain on the incorporation of ^{32}P inorganic phosphate into adenine nucleotides and guanine nucleotides of intact isolated rat diaphragm incubated in the absence of substrate The effect of epinephrine in a sodium free medium where sodium chloride isoosmotically has been replaced by choline chloride is also presented

( ) Number of experiments

Group Specific activity (cpm/μg P)	ATP*	ADP**	AMP	GTP*	GDP**	GMP
Ouabain (6)	589±60	344±15	46±6	824±90	882±87	69±8
Epinephrine (1 μg/ml) +ouabain (7)	1287±99	568±76	69±19	1275±100	1507±183	119±16
P	0 001	0 02	<0 10	0 005	0 005	<0 10
Epinephrine (0 2 μg/ml) +ouabain (3)	1363±50	516±80		1340±131		
P	0 001	0 02		0 02		
Control in choline chloride medium (4)	189±13	152±22	11±6			
Epinephrine (1 μg/ml) in choline chloride medium (4)	247±8	224±2	18±7			
P	0 005	0 02	<0 10			

* Refers to γ P of ATP and GTP

** Refers to terminal P of ADP and GDP

The effect of dibutyryl 3′ 5′ cyclic AMP on ^{32}P turn-over was investigated in additional experiments It was observed that incubation of the diaphragm in the presence of 0 5 mM dibutyryl 3′,5′ cyclic AMP gave rise to some activation of phosphorylase While dibutyryl 3′,5′ cyclic AMP increased phosphorylase *a* in the diaphragm from 15 % to 29 % (mean value of 5 expts) phosphorylase *a* was increased to a level of 38 % by epinephrine under identical experimental conditions In these experiments incubation was done for 30 min in the absence of added substrate

Dibutyryl 3′,5′ cyclic AMP also enhanced the ^{32}P labelling of ATP and ADP (Table IV), although the effect was less pronounced compared with the effect of epinephrine In the presence of epinephrine the specific activity of ATP and ADP was increased to an extent of 88 % and 51 % respectively (mean values) while the increase of ^{32}P labelling of ATP and ADP due to dibutyryl 3′ 5′ cyclic AMP amounted to 42 % and 19 % respectively (mean values) Incubation of the diaphragm with dibutyryl 3′,5′ cyclic AMP did not appear to have an effect on the specific activity of intracellular inorganic phosphate

Experiments on the dependence of the epinephrine effect on the ionic composition of the medium and on ion membrane transport have also performed The effect of epinephrine was therefore investigated in the presence of ouabain which is known as an inhibitor of the sodium potassium activated ATPase (Skou 1957) From Table V it is, however, seen that 0 2 mM ouabain did not have any effect on epinephrine stimulated ^{32}P incorporation into nucleotides either at high (1 μg/ml) or at low (0 2 μg/ml) concentrations of epinephrine

From the data reported in Table V it further appears that incorporation of ^{32}P into ATP was greatly depressed when sodium chloride in the medium isoosmotically was replaced by choline chloride As reported previously (Walaas *et al* 1969) there is no significant change in the specific activity of intracellular inorganic phosphate in the diaphragm incubated in choline chloride medium It is therefore conceivable that choline at this concentration inhibits mitochondrial phosphorylations Nevertheless a slight stimulating effect of epinephrine upon the ^{32}P labelling of ATP and ADP also was seen when the diaphragm was incubated in this sodium free medium

Discussion

The present work has shown that epinephrine as well as dibutyryl 3',5' cyclic AMP enhances the incorporation of ^{32}P inorganic phosphate into mononucleotides of the isolated intact diaphragm This indicates that epinephrine stimulates ^{32}P turnover into mononucleotides due to activation of adenyl cyclase An increased formation of cyclic 3' 5' AMP would give rise to stimulation of ATP requiring kinase reactions resulting in increased rate of mitochondrial phosphorylations of ADP which is formed The experimental conditions used, short incubation time and omission of added substrate during incubation would favour the demonstration of an epinephrine effect primarily on the membrane adenyl cyclase not too much influenced by secondary metabolic effects Somewhat different results have been reported by Hepp *et al* (1968) on the effect of epinephrine in isolated fat cells In these experiments epinephrine inhibited the incorporation of ^{32}P inorganic phosphate into ATP This may be due to the marked lipolytic effect by epinephrine on fat cells with subsequent inhibition of oxidative phosphorylations by free fatty acids as demonstrated by Wojtczak *et al* (1965)

The effect of dibutyryl 3' 5' cyclic AMP on the diaphragm differs from the epinephrine effect with respect to

a) The degree of phosphorylase activation

b) The extent of stimulatory effect of ^{32}P incorporation into ATP and ADP

c) Lack of increase of specific activity of intracellular inorganic phosphate

An activation of phosphorylase was established when the diaphragm was incubated in the presence of dibutyryl 3' 5' cyclic AMP, but the extent of activation was less than with epinephrine Similar observations have been reported by Lundholm *et al* (1969) by incubation of rat diaphragm in the presence of cyclic 3' 5' AMP A slow penetration of cyclic AMP and its derivatives may at least partly be responsible for this difference

It has also been shown that the stimulatory effect of dibutyryl 3',5' cyclic AMP on ^{32}P incorporation into ATP was less pronounced than the effect of epinephrine. Further the marked increase of specific activity of intracellular inorganic phosphate observed after epinephrine, could not be seen when the diaphragm was incubated in the presence of dibutyryl 3',5' cyclic AMP. The increased specific activity of intracellular inorganic phosphate due to epinephrine indicates an effect on membrane ion flux with increased exchange of phosphate across the membrane. It is therefore likely that under the influence of epinephrine but not dibutyryl 3',5' cyclic AMP, mitochondrial phosphorylations of ADP occur with inorganic phosphate of a higher specific activity. This may also be of importance for the more pronounced effect of epinephrine on ^{32}P labelling of ATP than after exposure to dibutyryl 3',5' cyclic AMP.

The effect of epinephrine but not of dibutyryl 3',5' cyclic AMP on phosphate exchange may indicate an epinephrine effect not involving activation of adenyl cyclase. The role of 3',5' cyclic AMP in regulating ion fluxes is still an unsettled question. In the perfused liver Friedmann and Park (1968) got evidence that the efflux of Ca^{++} and K^+ caused by *norepinephrine was mediated by 3',5' cyclic AMP. On the other* hand Lambotte (1968) reported that the loss of K^+ in the perfused liver under the influence of epinephrine occurred due to stimulation of β receptors and no correlation was found between the loss of K^+ and activation of phosphorylase caused by epinephrine. It has further been shown (Tsujimoto *et al.* 1965) that injection of 3',5' cyclic AMP into portal vein of dogs gives only a slight K^+ increase in serum compared with the effect of epinephrine. However both compounds gave the same hyperglycemic response and activation of liver phosphorylase. Recent observations by Anderson and Mohme Lundholm (1968) on smooth muscle give support to the concept that only the epinephrine effect upon α receptors is dependent upon the action of 3',5' cyclic AMP. The action of epinephrine upon β receptors is supposed to be related to potassium membrane permeability. The concept that epinephrine acts on different sites in the membrane, one at the level of the adenyl cyclase receptor complex and one associated with membrane ion fluxes, has got considerable evidence from the above mentioned observations as well as from the present work. Further work is however, necessary to establish the mechanism of action of epinephrine on membrane ion fluxes and the exchange of phosphate.

The present work gives no certain evidence however that the epinephrine effect on ^{32}P incorporation into mononucleotides is influenced by a change in the flux of sodium and potassium across the membrane mediated by the Na^+-K^+-Mg^{++} activated ATPase. The stimulatory effect of epinephrine was not inhibited by ouabain and a slight effect was also observed when the diaphragm had been incubated in a sodium free medium where sodium had been replaced by choline. It is therefore indicated that activation of adenyl cyclase by epinephrine is not much influenced by the distribution of sodium and potassium within the membrane. This is in accordance with the work by Lundholm *et al.* (1967) who studied the effect of epinephrine on the formation of cyclic 3',5' AMP in the diaphragm incubated in sodium free medium.

Although the content of cyclic 3',5' AMP in the diaphragm incubated in an all potassium medium was increased 10 fold, the increase of cyclic 3',5' AMP due to epinephrine was of the same order as in controls incubated in Krebs Ringer medium

In a recent publication (Walaas *et al* 1969) it has been reported that insulin exerts a stimulatory effect on the incorporation of ^{32}P into mononucleotides in the diaphragm, similar to the results reported here for epinephrine In contrast to the epinephrine effect this insulin effect is inhibited by ouabain and is abolished if sodium in the medium is replaced by potassium or choline It, therefore, appears that epinephrine and insulin promote increases in ^{32}P labelling of mononucleotides in the diaphragm by different mechanisms

We greatly [illegible] the excellent technical assistance of Mrs Genevieve Jones, Mrs Arnfrid Adler and [illegible] been given by The Norwegian Council for Science and [illegible] Fund and Grant No 275, North Atlantic Treaty Org[illegible] vision

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Pulmonary Gas Exchange as Influenced by G-Induced Shifts in Central Blood Volume

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Abstract

Rosenhammer, G *Pulmonary gas exchange as influenced by G induced shifts in central blood volume* Acta physiol scand 1970 78 404—409

Experimental observations are presented on interrelationships between G induced displace
cally significant correlation is demon
" and venous admixture in the lungs
displacement of blood volume from
s marked passive distension of the
dependent pulmonary blood vessels, and therefore in higher regional V/Q ratios, than if the G induced drain of blood out of the lungs is pronounced This explanation is supported by previous observations that G induced venous admixture increases when peripheral pooling of blood is counteracted by the action of the leg muscle pump

Pulmonary function changes with the transition from the recumbent to the upright body position (for review see Rosenhamer 1967) This change is closely associated with the hydrostatic effects on the circulatory system that result from the force of gravity acting along the long axis of the body Such hydrostatic effects can be exaggerated by using the human centrifuge and their consequence on pulmonary function can be studied in more detail In the centrifuge a test subject can be exposed to a gravitational force, or G force of variable strength and of arbitrary direction relative to the orientation of the body The increase of the G force in the head seat direction causes, through increased hydrostatic pressure gradients, a decrease the blood pressure in the upper parts of the body Also, the distribution of blood the circulatory system is altered so that there is a reduction of the amount of blo circulating in the intrathoracic spaces The venous return from below the level the heart is opposed by an increased hydrostatic pressure gradient and this, in tur results in a reduction of the cardiac output

When the G force acting in the head to seat direction is magnified the pulmona ventilation increases correspondingly (Barr 1963, Rosenhamer 1967) The respirat

ry stimulation probably occurs through the G-induced arterial hypotension and reduction of blood flow in the upper body parts, both by direct influence on the medullary respiratory centers and through reflexes from chemo- and baroreceptors (for review, see Rosenhamer 1967).

Since the circulatory changes involve a reduction of the intrathoracic blood volume, it appeared of interest to study whether in individuals exposed to the same increase of the G-force vector in the head to seat direction, a relation could be demonstrated between this reduction of blood volume and the ventilatory response If, for a given increase of the G-force, the drainage of blood away from the intrathoracic spaces is marked, then, through the resulting reduction in cardiac output and concurrent impairment of the arterial flow through the respiratory center and chemoreceptors, the increase in ventilation should also be relatively marked Furthermore, it appeared of interest to study during G-stress the possible existence of a relation between the total venous admixture in the lungs and the intrathoracic blood volume, the G effect on both variables showing considerable intra-individual variability (Rosenhamer 1967)

Methods

"Central blood volume" and pulmonary function were studied in 6 healthy male subjects (age 20—26 years) placed [illegible]
scription of the centr [illegible]
effects of the normal [illegible]
direction being unch [illegible]
with the subjective sensations experienced during centrifuge experiments

To demonstrate the potential relations discussed above [illegible]
to Hamilton *et al* (193 [illegible]
cutive determinations w [illegible]
3 G The technique em [illegible]
rections for sampling sy [illegible]

End tidal CO_2 tension was automatically obtained by a "breath by breath" gas sampling device (Brismar, Hesser and Matell 1962) The methods of calculation of the alveolar dead space, effective alveolar O_2 and total venous admixture were those used in previous studies (Barr 1963, Barr, Brismar and Rosenhamer 1969)

Results

Individual values for central blood volume, respiratory minute volume and venous admixture are shown in Table I The reduction in central blood volume in 7 subjects exposed to 3 G varied between 14 and 57 % of the value at normal gravity (range

TABLE I Calculated "central blood volume" (CBV, liters), venous admixture $\left(\frac{\dot{Q}_s}{\dot{Q}}, \%\right)$ and measured inspiratory minute volume (V_I, liters/min) at 1 G and 3 G

Subject	1 G			3 G[1]		
	CBV	V_I	$\frac{Q_s}{Q}$	CBV	V_I	$\frac{Q_s}{Q}$
1	1 9	12 5	5 8	1 2	18 6	41
2	2 7	10 8	8 3	1 2	19 3	43
3	1 6	9 9	6 9	1 3	16 6	95
4	2 0	7 1	7 3	1 6	25 9	67
5	2 2	8 6	3 6	1 4	15 3	25
6	2 9	10 3	5 5	2 5	18 9	75

[1] values obtained between the 5th and 8th min at 3 G

for reduction 0 2—1 5 l), while the simultaneous increase in ventilation varied from 6 to 14 l/min However, no significant correlation between the increase in ventilation and the change in central blood volume could be demonstrated in these experiments On the other hand, a significant correlation was obtained between the central blood volume reduction and the accompanying change in total venous admixture expressed as per cent of the cardiac output (Fig 1) Thus, it was found that those individuals whose venous admixture increased showed only small reductions in central blood volume while those with decreased venous admixture also showed comparatively large decreases in the central blood volume From the relatively small number of subjects studied, a correlation was obtained, shown in Fig 1, with a correlation coefficient of -0.92 ($P<0.01$)

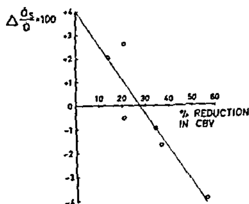


Fig 1 Relationship of individual changes in central blood volume and total venous admixture (in per cent of cardiac output) in subjects following an increase of the magnitude of the G force acting in the head-seat-direction from 1 G to 3 G $r = -0.92$ ($P<0.01$)

Discussion

Since no correlation was found between the changes in the central blood volume and pulmonary ventilation it seems to indicate that the G induced arterial blood pressure and flow reductions in the upper parts of the body, which may influence the respiration are not necessarily dependent on the accompanying caudad displacement of blood volume It can not be excluded nevertheless, from the present findings, that the above conditions obtained since the number of subjects studied was small and certain subjective factors may have influenced the respiratory responses

Furthermore, changes in the "central blood volume' do not merely reflect true blood volume changes but are also considerably influenced by the flow rate in the systemic arteries included in the "central blood volume' This effect becomes increasingly important the more peripherally the sample is taken In the present experiments with sampling from the radial artery, the increase of the G force vector in the head to seat direction from 1 G to 3 G in all likelihood caused a reduction of the arterial flow in the upper extremities (Rosenhamer 1967) Such a flow change increases the value for the central blod volume (Gleason *et al* 1959, Marshall and Shepherd 1961) The effect on this value of the actual G induced diminution of the blood volume in the thoracic regions is thus opposed by concurrent flow changes and this may have contributed to the absence of a demonstrable correlation between the reduction in ' central blood volume and the ventilatory response

The increase in G stress as studied here not only leads to a reduction of the total intrathoracic blood volume, but also to a redistribution of the blood that remains in the pulmonary circulation The blood tends to pool in the lower parts of the lungs the upper parts becoming more or less bloodless due to the increased effective weight of the blood

Barr (1963) has previously shown that with 5 G acting in the head to seat direction a large and statistically significant increase occurs in the venous admixture into the arterialized blood of the lungs (by about 15 % of the cardiac output) This can be explained as a result of the five fold increase of hydrostatic pressure difference between the upper and lower parts of the lungs, leading to congestion of the blood in the lower parts

However, in the present study, with 3 G acting in the same direction as in the above experiments no statistically significant change in venous admixture was obtained This difference between the results at 5 G and at 3 G can only partly be explained by the difference in magnitude of the G forces However a significant difference between the two experimental conditions was that G suits were used in the experiments at 5 G but not in those at 3 G The G suit opposes the G induced shift of blood into the lower portion of the body by a compression of this region. At normal gravity the G suit has been shown to increase both the total quantity of blood in the lungs (Bondurant, Hickam and Isley 1957) and the capillary blood volume as estimated by the $D_{L_{CO}}$ technique (Ross Maddock and Ley 1961)

Thus, the use of a G suit in the experiments at 5 G can be assumed to have caused a greater amount of blood to have remained in the pulmonary circulat[illegible]

otherwise have been the case In the dependent parts of the lungs the greater amount of blood probably resulted in a more pronounced passive widening of the blood vessels and a larger decrease of the ventilated alveolar spaces than would the smaller amount of blood without a G suit As a result lower ventilation/perfusion ratios *ie* a greater venous admixture could be expected By analogy it can be assumed that in the experiments at 3 G the size of the venous shunt would have been larger if a G suit had been used also in these experiments

Supporting evidence for the dependence of the venous admixture at a given G value on the total pulmonary blood volume was obtained by the observed correlation between a G induced reduction in the central blood volume and the simultaneous change in venous admixture

It might be argued that the flow dependence of the calculated "central blood volume may be responsible for the relation shown in Fig 1 However even if one assumes a reduction of the flow in the arteries of the arms in direct proportion to a G induced reduction in the cardiac output, there seems to be no obvious reason for such an effect since the venous admixture was expressed as per cent of the cardiac output Any correlation on the other hand between the reductions in cardiac output and central blood volume could not be demonstrated

In this context it seems appropriate to mention that on increasing the G-force from 1 G to either 3 G or 5 G a correlation between the individual increments in alveolar dead space and venous admixture could not be demonstrated This was the case whether the alveolar dead space was expressed in ml volume or as its index the arterial to end tidal CO_2 difference (*cf* Severinghaus and Stupfel 1957) and when the venous admixture was expressed in either *ml/min* as *per cent of the cardiac* output or as the index thereof the effective alveolar to arterial O difference It might have been expected that the impairment of perfusion and the resulting alveolar dead space in the upper parts of the lungs due to downward displacement of blood within the pulmonary circulation would result in a corresponding overperfusion and increase in venous admixture in the lower lung regions A significant reason for this not being the case was probably the individual differences in the concurrent downward drainage of blood away from the pulmonary circulation the more blood remaining in the lungs the greater would be the increase in the venous shunt The simultaneous increase of alveolar dead space would thereby be rather less pronounced

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Time Course of Anaphylactic Histamine Release and Morphological Changes in Rat Peritoneal Mast Cells

By

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Abstract

BLOOM, G D and N CHAKRAVARTY *Time course of anaphylactic histamine release and morphological changes in rat peritoneal mast cells* Acta physiol scand 1970 *78* 410—419

Anaphylactic histamine release and concomitant morphological changes were studied in rat peritoneal mast cells It was found that anaphylactic histamine release has a longer lag period than compound 48/80-induced release The time sequence of the morphological changes showed a close correlation to the histamine release These changes were essentially characterized by vacuolation granule alteration and granule extrusion Some peripheral granules were extruded with only minor changes The ultrastructural findings are consistent with a dual mechanism of histamine release—both intracellular and extracellular following granule extrusion

Previous observations using tissue fragments from sensitized guinea pigs showed that both anaphylactic histamine release (Schild 1939, Chakravarty 1960) and mast cell changes (Boreus and Chakravarty 1960) were initiated in 30 sec or less However histamine release and mast cell changes continued to occur up to about 10 min The guinea pig tissue preparation used in these experiments has its limitations—the diffusion barriers tending to prolong the time required for the penetration of the antigen into the deeper parts of the tissue and also for the diffusion of the released amine to the incubation medium Mast cells from rat peritoneal cavity being freely suspended in the peritoneal fluid, offer a better opportunity in this respect For the present study, peritoneal cell suspensions from sensitized rats were therefore used in order to follow the time course of histamine release, and the concomitant morphological changes in the mast cells as revealed by the light and electron microscope

Methods and Materials

Anaphylactic reactions were induced *in vitro* using mixed peritoneal cell suspensions from sensitized Wistar rats The method of sensitization and the general procedure for the collection of the cells have been described previously (Chakravarty 1968 a) Mixed peritoneal cells, of which usually 5—10 per cent are mast cells were pooled from 2 to 3 sensitized rats and

divided into 4—5 samples so that histamine release and morphological changes over the same time interval could be studied in samples of the same preparation To determine the state of sensitization of the individual rats, a drop of the cell suspension from each rat was incubated with the antigen and after fixation and staining with a solution containing formaldehyde and toluidine blue (see below), the mast cells were [illegible]

The pooled cells were suspended in Krebs- [illegible] ose and 1 mg/ml human serum albumin (final pH 7 0 [illegible] with the antigen, egg albumin, usually in 0 1 mg/ml [illegible] ere pre warmed at 37° C for 5 min and the incubation was continued after the addition of the antigen for periods varying from 5 to 60 sec The reaction was terminated at different time intervals for observations on histamine release by adding a 10 times higher volume of the chilled medium (0—4° C) and simultaneously transferring the tubes to an ice-cold water bath For electron microscopic studies 5 ml 4 per cent chilled (0—4° C) glutaraldehyde solution in 0 1 M phosphate buffer (pH 7 4) were added to 0 5 ml cell suspension after the different periods of incubation with the antigen The samples for histamine release were prepared as described [illegible] d on the guinea pig ileum [illegible] centrifuged at 0—4° C at [illegible] 2 hrs Thereafter the cells [illegible] and postfixed for 1 hr in [illegible] pH 7 4) Dehydration was [illegible] according to the method [illegible] ight and for an additional [illegible] knives Thick sections (~1 μ) were p [illegible] light microscopy Thin sections for [illegible] ate and lead citrate and were examir [illegible]

The time curves of anaphylactic as compared to compound 48/80 induced histamine release shown in Fig 1, were recorded from 6 expts in which cell suspensions from 1—2 Wistar or Sprague Dawley rats were used for each experiment Several samples of the same preparation were incubated with the releasers for 3—120 sec, after which the reaction was terminated as described above for the determination of histamine release To duplicate samples used for studying morphologi [illegible] d (0—4° C) fixing and staining [illegible] l and sodium chloride (Lagunoff [illegible] 3—120 sec) The cells were then [illegible] in the light microscope Mast cell changes in these preparations are shown in Fig 2 and 3

Results

As shown in Fig 1 a, histamine release induced by antigen antibody reaction was first detectable in 10—15 sec no release occurring 5 sec after exposure to the antigen in any of the 3 expts The ascending phase of the curve took 10—20 sec and the release was completed in 20 to 30 sec after contact with the antigen The experiments in Table I, in general agreement with these curves also show that there was no histamine release 5—10 sec after exposure to the antigen, while the release after 15 sec was still incomplete The histamine release by compound 48/80 was on the other hand initiated in only 3 sec and completed in 8 sec (Fig 1 b)—the release process thus being more rapid

The changes in the morphology of whole mast cells at different time intervals after exposure to antigen and compound 48/80 were characteristic and of the same pattern for both the releasers (Fig 2 and 3) The time course of the mast cell changes corresponded very well with the histamine release—the earliest changes being observed in 3 sec with compound 48/80 and in 10—15 sec with the antigen The changes were minimal at this period but proceeded rapidly to the full blown picture This was characterized by an irregularity of the margin of the cell with projecting granules Extrusion of granules was also observed The number of mast cells affected

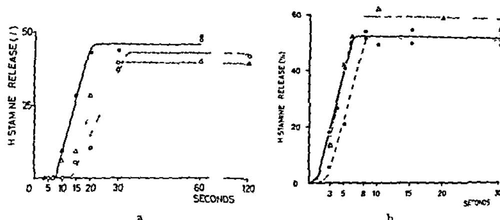


Fig 1 a Time course of anaphylactic histamine release from rat peritoneal mast cells *in vitro* (3 expts) Antigen (egg albumin) concentration 0 1 mg/ml There was no detectable spontaneous histamine release
b Time course of histamine release induced by compound 48/80 from rat peritoneal mast cells *in vitro* (3 expts) Concentration of compound 48/80 1—2 µg/ml The spontaneous histamine release (0—1 %) when detectable has been deducted

in this way varied depending on the state of the sensitization In an individual sensitized rat 30 to practically 100 per cent of the mast cells reacted to the antigen In the 48/80 experiments usually over 90 per cent of the mast cells reacted

Fixed epon-embedded and sectioned mast cells from sensitized rats incubated without antigen showed a completely normal appearance in the light as well as the electron microscope (Fig 4 a and 5) similar to that described previously (Bloom and Haegermark 1965 1967 Bloom Fredholm and Haegermark 1967) The anaphylactic reaction was produced *in vitro* with concentrations of antigen varying from

TABLE 1 Experiments for electron microscopy of sensitized mast cells 5—60 sec after exposure to the antigen

Exp	*Number of rats*	Antigen *egg albumin (mg/ml)*	Incubation time with *antigen (seconds)*	Histamine release (%)
1	2	0 1	60	17
2	3	0 1	60	21
3	3	0 1	10	0
		0 1	30	15
4	3	0 1	5	0
		0 1	20	30
5	3	0 1	15	21
		0 1	60	33

All rats were sensitized as judged from the mast cell reaction The degree of sensitization however varied

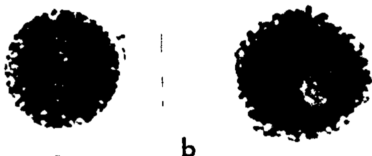


Fig 2 Anaphylactic mast cell changes Light micrographs toluidine blue stained a) Control mast cell incubated without antigen ×2700 b) Early changes in a mast cell 10 sec after contact with the antigen histamine release was just initiated ×2900

1 µg/ml to 1 mg/ml The minimal concentration which caused histamine release and mast cell changes was 5 µg/ml The highest concentration used 1 mg/ml caused very pronounced changes in a large number of mast cells The more moderate changes observed with the usual concentration of antigen, 0 1 mg/ml, are described below

As shown in Table I, incubation of sensitized peritoneal cells with antigen for 5—10 sec did not cause any histamine release No significant morphological changes in the mast cells were observed either However from 15—30 sec increasing amounts of histamine release were associated with characteristic changes in mast cell morphology (Fig 4 b—d 6—9) These changes were mainly seen in relation to the granules and may be summarized as follows

The affected granules appear to be altered in a characteristic manner They seem to swell somewhat and lose their electron dense, homogenous features Their matrix is thus revealed as a finely granular or filamentous material and furthermore a clear space of varying width separates this altered granule from the surrounding perigranular membrane The latter structure now delimits a granule containing vacuole from the cytoplasmic ground substance Frequently larger sacs are formed apparently by fusion and disruption of perigranular membranes In such membranous sacs several granules may appear (Fig 6—7) At the periphery of the cells either vacuoles

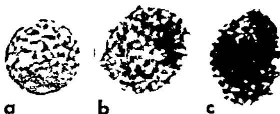


Fig 3 Mast cell changes induced by compound 48/80 (1 µg/ml) Light micrographs toluidine blue stained ×1900 a) Control mast cell incubated without releaser b) Early changes in a mast cell 3 sec after contact with compound 48/80, histamine release was just initiated c) Characteristic mast cell changes 5 sec after exposure to compound 48/80

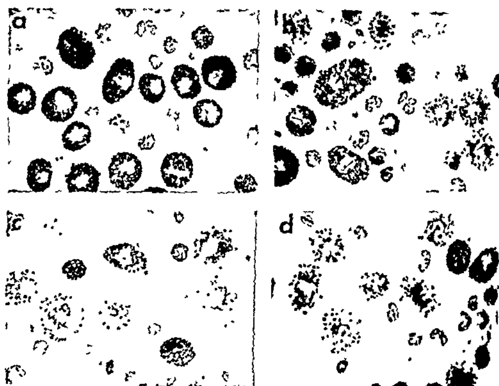


Fig 4 Light micrographs of Epon sections stained with toluidine blue ×800 Sensitized rat peritoneal cells incubated at 37° C without antigen (a) and with 0 1 mg/ml antigen for 12 sec (b) 20 sec (c) and 60 sec (d) Histamine release 21—33 % with antigen (b—d) not detectable in control (a)

containing single granules or membranous sacs containing several granules may be seen to open up expelling their contents to the extracellular space (Fig 6—9)

Alteration of the granules appears to be a stepwise process as not only does the electron density vary among granules within the same cell but also within various zones of the same granule (Fig 6 and 8) It is within these electron lucid altered granules or zones that the fine texture of the granule matrix is clearly seen It is interesting that in spite of the lack of a delimiting membrane many of these altered granules remain fairly intact and their contours are rather sharply defined (Fig 7—8) even after expulsion from the cells

In some mast cells however even those incubated for as long as 60 sec, peripheral granules adjacent to the cell membrane are expelled without or with only slight changes in granular fine structure As seen in Fig 9, even this granule release appears to be preceded by the formation of clearly visible vacuolar structures around the granules An interesting feature especially noted in this type of reaction, is a fuzziness in contours of the released granules This decreased sharpness of the granule edges is due to a finely filamentous material which appears to radiate from the granu matrix (Fig 9, inset)

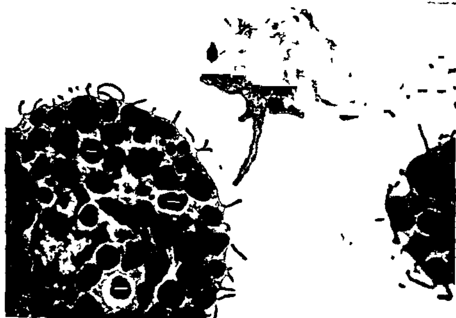


Fig 5 Peritoneal cells from a control specimen (sensitized cells incubated for 60 sec at 37 C without antigen) Parts of two mast cells and an eosinophil leukocyte are seen The mast cell granules show somewhat varying sizes and shapes The majority are electron dense and intimately surrounded by perigranular membranes Three granules in different stages of alteration are observed (—) Electron micrograph. ×7700



Fig 6 Sensitized rat peritoneal mast cell after incubation with 0 1 mg/ml antigen for 20 sec A fair number of altered granules appear either single granules in vacuoles or several together in larger sac like formations At the periphery of the cell released granules as well as granules in the process of being expelled are seen Note the varying electron density of the matrix altered granules Arrow indicates a zonal difference in electron density within a single Electron micrograph ×7200

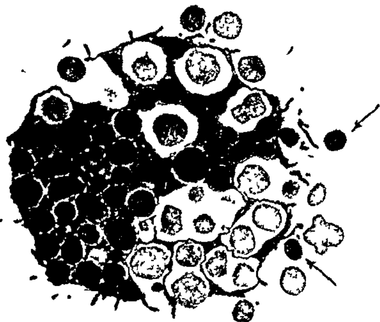


Fig 7 Electron micrograph of a sensitized rat peritoneal mast cell incubated with 0.1 mg/ml antigen for 20 sec. Numerous vacuoles and membranous sacs containing altered granules are observed. Some of these structures are apparently located deep within the cells. Granules which have been released or are in the process of being released are seen. While most released granules are characteristically altered some are not (arrows). × 7200

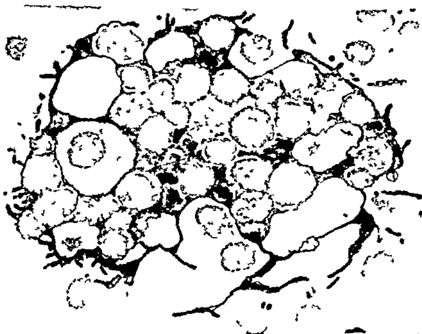


Fig 8 Grossly affected rat peritoneal mast cell sensitized and incubated with 0.1 mg/ml antigen for 20 sec. The granules show a wide spectrum of alterations. Many extracellular altered granules are seen as are cytoplasmic vacuoles and sacs which appear to be expelling their granular contents. Electron micrograph × 8850

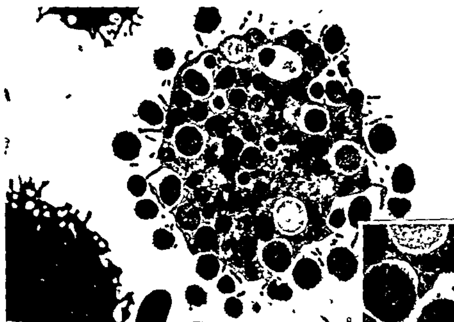


Fig 9 Sensitized rat peritoneal mast cell incubated with 0 1 mg/ml antigen for 10 sec Note numerous released granules which are fairly electron dense and which show unsharp contours. In the cytoplasm vacuolation and granule alteration are taking place ×7700 Inset Parts of granules in different stages of alteration The fuzzy appearance of the granule in the process of being released is caused by strands of a filamentous material radiating from the granule Electron micrograph ×15 000

Discussion

Peritoneal mast cells from rats, actively sensitized with egg albumin and hemophilus pertussis vaccine, release their histamine upon incubation *in vitro* with egg albumin used as antigen The time course of this release shows a general similarity to that obtained upon incubation of rat peritoneal cells with certain histamine releasers such as compound 48/80 and bee venom (Bloom Fredholm and Haegermark 1967) However, there is a difference in the initial phase of the reaction as the lag period is much longer for the anaphylactic release as compared to that induced by 48/80 and bee venom As shown in Fig 1 and Table I the anaphylactic histamine release was initiated in 10—15 sec and completed in 20—30 sec In contrast compound 48/80 induced release was initiated in 3 sec and completed already in 8 sec, by which time anaphylactic release had not even started (Fig 1)

Any attempt to interpret this characteristic lag phase in anaphylactic histamine release must necessarily be speculative as practically nothing is known about the nature of the antigen antibody reaction in mast cells Although it has been shown that such a reaction does take place in isolated mast cells (Mota and Dias da Silva 1960) it is still not clear if it occurs at the cell surface or intracellularly (Humphrey and Mota 1959 Mongar and Schild 1962) Unfortunately, this question cannot be answered by the present studies either One can assume, however that there

specific antibody sites in the mast cell with which antigen must combine to initiate the chain of reactions that finally leads to histamine release This could explain the relatively longer lag period for histamine release induced by antigen as compared to that by compound 48/80 which probably exerts its effect over the entire cell membrane It has recently been shown that mast cells can phagocytize particulate matter (Padawer and Fruhman 1968 Padawer 1969) Phagocytosis of protein by the cells has also been reported (White *cit* Mongar and Schild 1962) If antigen is incorporated into the cells before reacting with antibody this would also tend to prolong the lag period

The ultrastructural changes in antigen treated mast cells observed in the present study are very similar to those observed by Bloom and Haegermark (1965) after treatment of the cells with compound 48/80 Similar findings were also reported by Chakravarty, Gustafsson and Pihl (1967) However, the Ruthenium red technique used in the latter experiments while staining the polysaccharide material of the cells was inadequate in revealing the fine structure of granules and cellular membranes

The extrusion of granules with minimal signs of alteration in their structures (Fig 7 and 9) is an interesting observation The relatively high electron density of these granules and the fuzzy appearance of their contours caused by radiating strands of fine filamentous material suggest that they may be in the process of losing some granular material It seems likely that these granules have been expelled from the cells before the alterations generally seen intracellularly (Fig 6—8) have had time to occur

We have tried to correlate our morphological findings with the histamine release curves Qualitatively there is a very good correlation between the initiation of mast cell changes and of histamine release During the ascending phase of the histamine release curve the number of mast cells showing characteristic changes increases However when the peak of histamine release has been reached there may be a divergence between percentage of histamine release and of mast cells showing morphological changes For instance a 90 per cent reaction of the mast cells may be associated with only 20 per cent histamine release In contrast to the guinea pig mast cells (Mota and Vugman 1956) rat peritoneal mast cells do not disintegrate entirely after exposure to either antigen or compound 48/80 (Chakravarty 1969) One may therefore assume that the amount of histamine released from rat peritoneal mast cells depends both on the degree of changes in these cells and the number of granules expelled

It is interesting to note that mast cells under the influence of histamine releasers— antigen or compound 48/80—show structural changes in granule alteration not only at the periphery of the cells but also deep within the central parts of the cytoplasm (Fig 6—9) This raises the important question if histamine is released intracellularly or extracellularly after granule extrusion It has been postulated by Thon and Uvnas (1967) that histamine release by compound 48/80 from mast cells is a two step process requiring the initial extrusion of granules However, Carlsson and Ritzen (1969) utilizing microspectrofluorometry and interference microscopy have

presented evidence for amine release (5 HT) from mast cells without concomitant granule release This was shown both in response to compound 48/80 and antigen antibody reaction

On the basis of the ultrastructural studies carried out in our present and previous experiments it appears likely that histamine release can take place both intra- and extracellularly The altered appearance of cytoplasmic granules (Fig 6—8) may reflect an intracellular release, possibly due to permeability changes in the peri granular membranes On the other hand, some peripheral granules adjacent to the cell membrane, which are extruded in a relatively unaltered form (Fig 9) may well transfer their load of histamine—following extrusion—to the extracellular fluid

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The skilful technical assistance of Miss Marianne Borg and Miss Ingrid Olofsson is gratefully acknowledged

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Effect of Chlorpromazine, Mepyramine, Prenylamine and Reserpine on 5-Hydroxytryptamine Content and Fine Structure of Rat Peritoneal Mast Cells Incubated in Vitro

By

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Abstract

JANSSON, S.-E. *Effect of chlorpromazine, mepyramine, prenylamine and reserpine on 5-hydroxytryptamine content and fine structure of rat peritoneal mast cells incubated in vitro.* Acta physiol. scand. 1970. 78. 420—430.

The mechanism of action of the drugs tested in releasing 5-hydroxytryptamine from mast cells in vitro was studied under different incubation conditions and by observing the morphological changes associated with the amine release. It was proposed that chlorpromazine, mepyramine and prenylamine release 5-hydroxytryptamine by a mechanism of action not requiring energy because neither did the drugs exert their maximal effect at physiological pH values nor was the release blocked by pre-incubation with sodium cyanide. The suggestion is supported by the morphological observations revealing destructive changes of the mast cells. The reserpine-induced release of 5-hydroxytryptamine from mast cells was small upon incubation in a phosphate buffer and was completely inhibited upon incubation in a physiological medium containing divalent cations and glucose. The release was not decreased by lowering the temperature of the incubation medium from 37° C to 23° C which indicate a mechanism of action not requiring energy. On the other hand reserpine had a maximal releasing effect around neutral pH values and the release was totally blocked by pre-incubation with sodium cyanide suggesting a necessity for metabolically active cells. An energy-requiring mechanism of action of reserpine was further supported by the observed morphological alterations which were much of the same nature as those induced by compound 48/80.

In a previous paper it was shown that chlorpromazine and prenylamine were effective in releasing 5-hydroxytryptamine (5-HT) from mast cells *in vitro* (Jansson and Penttilä 1969). It was also shown that mepyramine and reserpine released small but significant amounts of 5-HT. Moreover, it was observed that the release induced by mepyramine and reserpine took place without significant degranulation, while the cells were completely disintegrated by chlorpromazine and prenylamine.

In contrast to these results, Moran *et al.* (1962) did not observe any release of 5-HT by reserpine from mast cells *in vitro*, while Van Orden *et al.*, (1967) who studied tumour mast cells *in vitro* reported a significant release which increased with the incubation time.

Because of these discrepancies concerning the release of 5 HT and in a view of our incomplete knowledge of the mode of action of drugs on the mast cell amines (Bloom and Haegermark 1967), the effects on the mast cell 5 HT of chlorpromazine, mepyramine prenylamine and reserpine was studied *in vitro* under different incuba ion conditions

Material and Methods

Mixed peritoneal cells of which mast cells constituted about 3 % (Jansson *et al* 1967) were used in the present study About 200 rats, of the Sprague Dawley strain 150—300 g were ghtly anesthetized and bled The peritoneal cells were washed out with 10 ml of 0 15 M [illegible]

[illegible] liconized

Incubation technique

The incubations were performed in 8 ml siliconized culture tubes in a water bath under agitat on Before incubation, the cells were prewarmed for about 15 min at the incubation temperature Thereafter 0 10 ml of the drug to be tested dissolved in 0 15 M phosphate buffer was added to 0 90 ml of the cell suspension When the effect of inhibitors was tested the cells were pre incubated with the inhibitor for 30 min at 37° C before the drug was added Unless otherwise mentioned the incubations were interrupted after one hour by dilution with ice-cold phosphate buffer and they were then sedimented by centrifugation at 1500×g for 10 m n.

Determination of 5 HT

The 5 HT content of this sediment was determined by a semi micro modification of the [illegible]

[illegible] The release of 5 HT was estimated by expressing the 5 HT content of the cell sediment in per cent of the 5 HT content of a sediment (set at 100 %) of a sample incubated under ident cal conditions but without added drug The release of 5 HT in samples made for electron microscopy was calculated by determining the released amount of 5 HT in the super natant.

The significance of the mean differences was estimated using Student s t test or analysis of variance (Eranko 1955)

Electron microscopy

The cell sediment obtained by centrifugation at 1500×g was fixed by resuspending it in 4 % glutaraldehyde in 0 15 M phosphate buffer pH 7 3 for 1 hr at 4° C After fixation the cells were spun down at 4° C at 1500×g rinsed in 0 3 M sucrose pH 7 3 and postfixed in 1 % OsO_4 in 0 15 M phosphate buffer pH 7 3 for 2 hrs Sections were obtained using a Porter Bloom ultramicrotome and they were stained with uranyl acetate and/or lead citrate Electron micrographs were taken with a Philips EM 200 electron microscope

Reagents

The reagents used in analyses were commerciable available analytical or reagent grade products prepared according to Weissbach (1961) The following chemicals were used Chlorpromazine (Medica Oy Ab) Prenylamine (Segontin® R. Hoechst AG), Mepyramine (Ori n Oy Reserpine (Serpasil® Ciba)

Results

Effect of incubation temperature

The effect of the temperature of the incubation medium on the release of 5 HT from mast cells by the drugs tested was studied at 0° C, 23° C and 37° C The results a

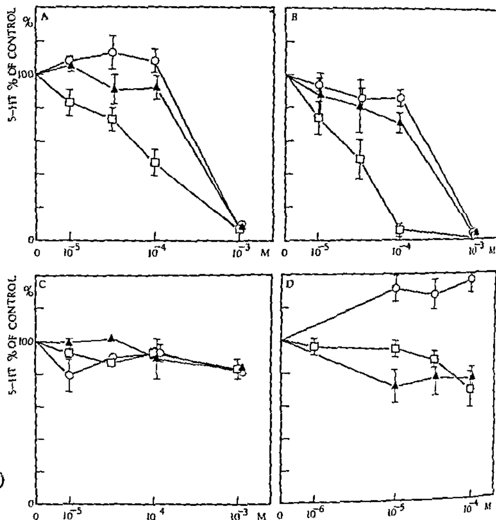


Fig 1 The effect of temperature on the release of 5 HT from rat peritoneal mast cells in vitro by different concentrations of chlorpromazine prenylamine mepyramine and reserpine In cubations were carried out for one hour in a 0 15 M phosphate buffer pH 6 9 The ordinate gives the 5 HT content of cells incubated with drugs in per cent of the 5 HT content of a sample incubated in pure phosphate buffer and set at 100 % Means and standard errors of 3—11 experiments

A Chlorpromazine B Prenylamine, C Mepyramine D Reserpine

○ 0° C, ▲ 23° C □ 37 C

presented in Fig 1 Chlorpromazine and prenylamine released 5-HT in increasing amounts with increasing temperature and at increasing concentrations At 10^{-4} M chlorpromazine released about 50 %, while the prenylamine induced release was almost 100 % Chlorpromazine showed a slight statistically non significant tendency to inhibit the spontaneous release at 0° C Like prenylamine chlorpromazine induced an almost quantitative release at 10^{-3} M regardless of the incubation temperature

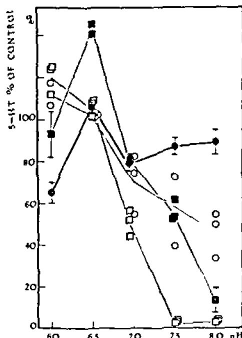


Fig 9 Effect of pH on the release of 5 HT from rat peritoneal mast cells in vitro by chlorpromazine prenylamine, mepyramine and reserpine Incubations were carried out for one hour at 37° C in a 0 15 phosphate buffer at the pH desired The release was calculated by expressing the 5-HT content of experimental samples in per cent of controls incubated at the same pH For the concentrations of the drugs see text Each point represents the result of one experiment or the mean and standard error of 4—7 expts
● Reserpine 10^{-4} M ○ Mepyramine, 10^{-3} M □ Chlorpromazine 5×10^{-5} M, ■ Prenylamine 3×10^{-5} M

At 10^{-5} M chlorpromazine and prenylamine released about 15 % and 25 %, respectively The morphological alterations of the mast cells were small and consisted chiefly of granular alterations (Fig 8) indicating that degranulation of the mast cells is not yet discernible after such a small amine release After incubation at 5×10^{-5} and 10^{-4} M definite changes were seen although the amine loss was still 30 % and 50 % only The cells showed large vacuoles or were totally disrupted The nucleus was swollen or amorphous and the nuclear membrane was fragmented The granules were swollen and the perigranular membranes were absent or broken The electron density of the granules was decreased revealing a granular internal structure Degranulation and disruption of the mast cells was extensive at both concentrations when the incubations were performed at 37 C (Fig 10 but not at 0° C when the cells were mostly intact but filled with swollen and pale granules (Fig 11) The marked granular alterations do not correlate with the slight (−10 % to +10 %) release of 5 HT at this temperature

The pronounced morphological and biochemical alterations after incubation with chlorpromazine or prenylamine were in sharp contrast to the results obtained upon incubation with mepyramine or reserpine At 0° C reserpine retarded the release of 5-HT causing an increase of the 5-HT content 30 % above the control level at all concentrations tested (Fig 1) No morphological alterations were observed The reserpine induced 5 HT release was slight even at 37° C (Fig 1) At 23° C about 25 % was released regardless of the concentration At 10^{-4} M and 5×10^{-5} M reserpine released at 37° C 30 % and 13 % respectively which was found to

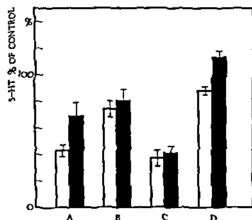


Fig 3 The effect of the addition of glucose and calcium and magnesium ions to the incubation medium on the release of 5 HT from rat peritoneal mast cells in vitro by chlorpromazine, mepyramine prenylamine and reserpine Incubations were carried out for one hour at 37° C in a modified Krebs salt solution supplemented with glucose and calcium chloride The release of 5 HT was expressed in per cent of controls incubated in Krebs salt solution without added drugs and compared with the release in samples simultaneously incubated in 0 15 M phosphate buffer Means and standard errors of 6—7 expts
A Chlorpromazine B Mepyramine C Prenylamine, D Reserpine

statistically significant The morphological alterations consisted chiefly of granular alterations (Fig 12)

Like reserpine, mepyramine induced only a slight 5 HT release (Fig 1) After incubation in 10^{-3} M mepyramine at 37° C, only 17 % was released Smaller concentrations did not cause any significant effect on the 5 HT content

Effect of pH

When the effect of the pH of the incubation medium was studied such a concentration of each drug was chosen which was predicted to induce a release of about 30 % at pH 6 9 However, in these experiments 5×10^{-5} M chlorpromazine released about 50 % The results are presented in Fig 2 As can be seen all drugs but reserpine released far more 5-HT in an alkaline solution than in a neutral or acidic one An energy dependent mechanism in the amine release with the optimum pH at about 8 0 is unlikely Thus the effect of alkalinization is probably due to nonspecific damage Reserpine on the other hand, did not show such a great depletion effect at the alkaline pH

Effect of ions and glucose

The release induced by mepyramine and prenylamine was the same when the incubation was performed in Krebs salt solution supplemented with glucose and calcium chloride (Eranko and Raisanen 1966) or in a 0 15 M phosphate buffer made up of Na_3PO_4 and Na_2HPO_4 (Fig 3) Chlorpromazine and reserpine on the other hand, released considerably less 5 HT in Krebs salt solution than in the phosphate buffer It was observed that the presence of excess K^+ ions in the medium depressed the reserpine induced release (Fig 4) The concentration of K in the Krebs salt solution was not however high enough to explain the observed retardation of the reserpine induced release in this solution Addition of 2 % human serum to the incubation medium did not significantly affect the release in any respect

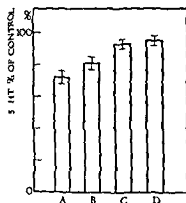


Fig 4

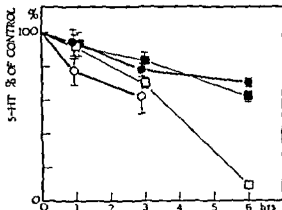


Fig 5

Fig 4 Effect of the concentration of Na and K ions on the reserpine induced release of 5 HT from rat peritoneal mast cells in vitro Incubations were carried out for 1 hr at 37° C in 0 15 M phosphate buffers made up of NaH_2PO_4 Na_2HPO_4 and KH_2HPO_4 K_2HPO_4 to give different Na and K ion concentrations The release of 5 HT was calculated by expressing the 5 HT content of reserpine incubated samples in per cent of the 5 HT content of samples incubated in respective buffer Means and standard errors of 4—10 expts
A Only Na B Na /K = 28/1 C Na^+/K = 2 8/1 D Only K

Fig 5 Effect of the incubation time on the reserpine induced release of 5 HT from rat peritoneal ma[illegible]
salt solution s[illegible]
at 37° C Th[illegible]
incubated n[illegible]
standard error of 3—6 expts
☐ Control phosphate buffer ■ Control Krebs Ringer ○ Reserpine 10^{-4} M phosphate buffer
● Reserpine 10^{-4} M Krebs Ringer

Effect of incubation time on the reserpine induced release

By prolonging the incubation time to 6 hrs the 5 HT content of the control samples incubated in a phosphate buffer steadily decreased to below 10 % of original value while samples incubated in the Krebs Ringer glucose solution showed a decrease of about 40 % (Fig 5) Regardless of the nature of the incubation medium 10^{-4} M reserpine did not cause a significant loss of 5 HT even after 6 hrs incubation

Effect of pre incubation with sodium cyanide

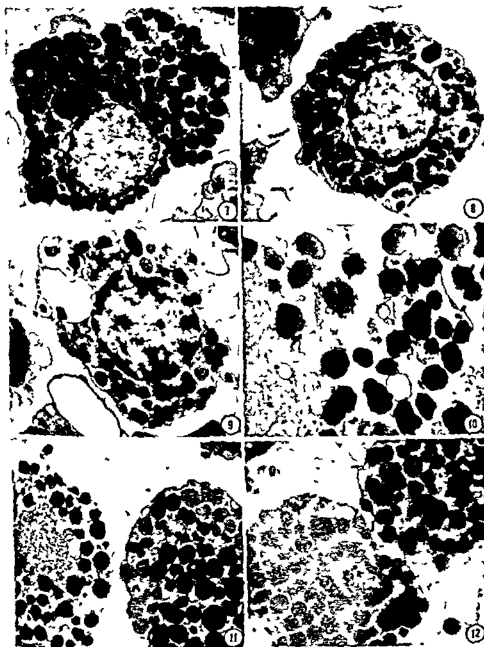
Pre incubation with sodium cyanide was carried out at 10^{-4} M for 30 min at 37 C (Table I) As [illegible]
sodium cyanid[illegible]
prenylamine T[illegible]
experiments were carried out with reserpine the mean release being [illegible]
control Pre incubation with NaCN almost completely prevented the release [illegible]
mean depletion being 2 % of original The differences between the means obt[illegible]
without and with NaCN was statistically significant ($p<0\ 001$)



Fig 6 Electron micrograph of a rat peritoneal mast cell incubated for 1 hr at 37° C i 0.15 M phosphate buffer pH 6.9 Note the highly osmiophilic granules which are surroun by perigranular *membranes* and evenly distributed within the cytoplasm An unusually w developed endoplasmic reticulum is to be seen to the right of the nucleus At the periph somewhat clumpsy cytoplasmic protrusions can be seen Magnified to 15 000 × from an orig low power electron micrograph

TABLE I Effect of Pre incubation with NaCN on the Drug Induced Release of 5 HT from R Peritoneal Mast Cells in Vitro Pre incubation was carried out at 37° C for 30 min 10⁻⁴ M The release is expressed in per cent of controls incubated with or without NaC respectively Between the two groups of controls was no significant difference Means an standard deviations

Drug	Conc (M)	Number of tests	Release of 5 HT % (±S D) Control	NaCN	p<
Chlorpromazine	10^{-5}	2—2	16.2	17.2	
	5×10^{-5}	2—2	28.1	25.8	•
Prenylamine	10^{-5}	2—2	13.0	22.0	••
	5×10^{-5}	2—2	29.6	25.4	•
Mepyramine	10^{-5}	2—2	9.0	13.1	
Reserpine	10^{-4}	7—7	28.2 (±12.4)	2.1 (±3.9)	0.001



F g 7 A control cell n ubated in phosphate buffer for 1 hr at 0 C show ng essentially the same features as Fig 6 × 00

Fig 8 Electron mic ograph of a mast cell incubated for 1 hr w th 10 ᵃ M chlorpromazine at 37° C To the right t the cell two swollen granules are to be seen surrounded by peri granular halos A small vac o containing amorphous granular material is to be seen at the lower cell periphery × 7 00

Discussion

The effect of chlorpromazine and prenylamine on the 5 HT content of mast cel' was not temperature dependent at high drug concentrations Around 10^{-4} M ther were, however, differences between the released amounts of 5 HT at 0°, 23° an 37° C It was therefore investigated if there was any energy requiring mechanis behind the observed temperature dependant release It was found that if there is a active mechanism it exerts its maximal effect around pH 8 0, which seems im probable The addition of glucose to the incubation medium did not either accelerat the release Further, the release was not opposed by pre incubation with NaCN These results suggest a mechanism of action not requiring energy of chlorpromazin and prenylamine in releasing 5 HT from mast cells This suggestion is supported b the observation that both drugs have lytic properties (Ahtee 1966, Grobecker 1968)

The retarded release at 0° C can be explained according to Grobecker et al (1968) who has observed that when adrenal medullary granules are incubated in th presence of prenylamine at low concentrations the granules tend to swell and there by the amines are prevented from diffusing out because of the enlargement in sur face area/volume ratio It will be emphasized that in the present study the mas granules did swell upon incubation at 0° C with prenylamine or chlorpromazine at high concentrations

Because of the slight release induced by mepyramine it was difficult to investigate the nature of the release mechanism Anyhow, it seems clear that the release was neither temperature dependent nor opposed by pre incubation with NaCN The observations suggest a mechanism which does not require energy

In a previous paper it has been reported that reserpine releases small but signif cant amounts of 5 HT from mast cells (Jansson and Penttila 1969) It was thought that the acute nature of the incubation conditions (1/2 hr at 23° C) prevented a more pronounced release In the present study the incubation time was prolonged to 6 hrs at 37° C but further amounts of 5 HT was not released as compared to the

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Fig 9 Electron micrograph of a mast cell incubated for 1 hr with 5×10^{-5} M chlorpromazine at 37° C The 5 HT loss is about 43 % Taking into account the incubation the cell is in a rather good condition The nucleus is unaltered but cytoplasmic vacuols of different sizes can be seen containing swollen and pale granules Note the amorphous remnants of a leucocyte to the left of the picture × 7700

Fig 10 Electron micrograph of extracellular mast granules after incubation with 10^{-4} M prenylamine at 37° C The 5 HT content is 20 % of normal Nuclear fragments are to be seen in the lower left corner The granules are swollen and of decreased electron density reveaing an internal granular structure Most of the granules are non delimited and only fragments of perigranular membranes are to be seen × 11 900

Fig 11 Electron micrograph of two mast cells incubated with 10^{-4} M chlorpromazine for 1 hr at 0° C The 5 HT content was about normal despite the high drug concentration The slight release does not either correlate with the swollen granules some of which reveal a granular internal structure The cell to the left is degranulated and has an amorphous nucleus but in act cells were more common × 7700

Fig 12 Electron micrograph of mast cells incubated for 1 hr with 10^{-4} M reserpine at 37° C Many of the intracellular granules are swollen and some are of decreased electron density Perigranular halos are to be seen in the lower cell 5 HT content about 63 % of normal × 9100

release at 1 hr The addition of serum to the incubation medium did not either enhance the release in the way it has been found for compound 48/80 (Uvnas and Thon 1959)

Although the releasing effect of reserpine on blood platelet 5-HT is well documented the mechanism is still speculative (Paasonen 1965) In the present study some diverging observations were made No strict temperature dependance at 37° C was observed nor was the release accelerated by the addition of glucose to the incubation medium These observations suggest a non energy requiring mechanism This suggestion is supported by results indicating that reserpine has lytic properties (Grobecker 1968)

Even if a mechanism not requiring energy seems convenient there are some observations that make it doubtful First, all drugs tested but reserpine released far more 5 HT at alkaline than at neutral or acidic pH values This indicates not an active energy requiring mechanism with a maximum around 8 0, but probable an enhanced diffusion of the alkaline 5 hydroxyindole from mast cells deteriorated by challenge with drug and unphysiological pH Reserpine, on the other hand, released somewhat smaller amounts at alkaline values than at neutral There seems to be in other words a mechanism underlying the reserpine induced release of 5 HT from mast cells which has a maximum around neutral pH values Whatever this mechanism could be, it was found, that it was blocked by pre-incubation with NaCN

As was pointed out in the introduction, Moran and Westerholm (1962) did not observe any effect of reserpine in vitro on the mast cell 5-HT content, while the present author found a small but significant effect The discrepancy can obviously be explained by the present observation that reserpine had no decreasing effect on the 5 HT content upon incubation in Krebs Ringer solution which is essentially of the same composition as the incubation medium used by Moran and Westerholm.

The present morphological results give further information about the action mechanism of the drugs tested At small concentrations chlorpromazine and prenylamine released small amounts of 5-HT with minor granular alterations, including formation of perigranular halos At higher concentrations however, both drugs disrupted the cells and affected also the cell nucleus and membranes to a high degree even if the amine loss was below 50 % The alterations resembled the results obtained with n decylamine which has been shown to liberate histamine from mast cells by a mechanism which does not require energy (Bloom and Haegermark 1967) Reserpine caused, on the other hand, alterations which have much in common with those induced by incubation with compound 48/80 including a pronounced tendency for a formation of perigranular halos but leaving the cell nucleus largely unaffected (Bloom and Haegermark 1965 Singleton and Clark 1965) It will be pointed out that compound 48/80 has been shown to release mast cell amines by an energy requiring mechanism (Uvnas 1961, 1963, 1967, Uvnas and Thon 1961)

The electron microscopic part of the present study was carried out in the Electron Microscopic Laboratory, University of Helsinki The skilfull assistance of Mr M Nyholm is gratefully acknowledged

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Is the Tonic Stretch Reflex Dependent upon Group II Excitation?

By

S Grillner

In a recent article P B C Matthews (1969) concluded that the secondary endings of the muscle spindle contribute autogenetic excitation in the tonic stretch reflex of the soleus muscle in the decerebrate cat One factor of crucial importance for the above conclusion shall be considered below

Low amplitude longitudinal vibration of the soleus muscle gives a selective activation of the primary endings (Ia afferents) and provided that the muscle is fairly extended each afferent, is driven with a frequency set by the vibrator (Brown Engberg and Matthews 1967) Matthews measured the reflex tension developed in the muscle at different frequencies of vibration From these results he deduced that the increase in discharge rate in the Ia afferents with one impulse/sec increased the tension with a few g wt (3 9 g wt /Hz in Fig 2 B of Matthews 1969) The gain of the stretch reflex calculated in g wt /mm extension is in the order of 90 g wt /mm (cf Matthews 1969 Fig 2 A) Since the sensitivity of the primary endings to extension is 2—5 imp/sec/mm (for references see Matthews 1969), the gain can also be expressed as 45—18 g wt /Hz The discrepancy between the gain factors obtained with vibration (3 9 g wt /Hz) and with extension (45—18 g wt /Hz) led Matthews to postulate that *the primary endings cannot be solely responsible for autogenetic* excitation in the stretch reflex This postulate rests on the assumption that the "gain" in g wt /Hz obtained with vibration and with extension are directly comparable However, this is not the case since the latter depends not only on the increased tension due to recruitment of new motor units but also on the length tension relationship of the contracting muscle fibres

The length — tension curve of the contracting *deafferented muscle is within a* very large range (>10 mm) approximately rectilinear and the slope can be in the order of 115 g wt /mm for a maximal contraction in the soleus muscle (calculated from Fig 8 of Rack and Westbury 1969, the data were obtained with distributed stimulation at physiological frequencies of the different ventral root filaments supplying the muscle) This value (115 g wt /mm) is the maximal that the muscle can account for, the actual value would presumably be roughly proportional to the percentage of muscle fibres active In order to estimate the tension (g wt /mm) contributed by the recruitment of new motor units in a reflex contraction, the gain o

the active muscle fibres (g wt /mm) should be subtracted from the gain of t stretch reflex (g wt /mm) The values thus obtained can be expressed in g wt/I and compared with the value obtained with vibration

It follows that Matthews' calculations regarding the stretch reflex in terms "required Ia sensitivity in imp/sec/mm" is not relevant for the comparison of stret and vibration It should here also be noted that the values of the gain factor wi vibration (g wt /Hz) would be expected to be somewhat low due to the type stimulation used, the vibratory stimulus as such applied to a contracting muscle ma also have other effects (*cf* Grillner 1969, p 24)

Matthews has used the lack of occlusion between the tonic stretch reflex and th vibratory response to support the "postulate of group II excitation (Fig 3 and 4 Matthews 1969) A vibration response (200 Hz) is superimposed on a tonic stretc reflex at a given length and then the muscle is further extended to give a stretc reflex with the amplitude of the previous total response (vibration + tonic stretc reflex) Matthews then states 'If the Ia fibres were the sole origin of the stretc reflex one would conclude that the larger extension by itself excited the Ia fibres a about 200 Hz' This argument completely ignores the tension added by extensio of the contracting muscle fibres and is thus not valid Since the sensitivity to exten sion of Ia fibres is comparatively low (*cf* above), one would rather expect a ver limited degree of occlusion with the vibratory response and this can also be observed in some of Matthews' records, in addition the more efficient contraction at higher levels of extension can mask some occlusion

The above considerations show that the results obtained by Matthews do not warrant the conclusion that the secondary endings contribute excitation in the tonic stretch reflex

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The Effect of Asphyxia upon the Lactate, Pyruvate and Bicarbonate Concentrations of Brain Tissue and Cisternal CSF, and upon the Tissue Concentrations of Phosphocreatine and Adenine Nucleotides in Anesthetized Rats

By

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Received 3 June 1969

Abstract

KAASIK, A. E., L. NILSSON and B. K. SIESJÖ. *The effect of asphyxia upon the lactate, pyruvate and bicarbonate concentrations of brain tissue and cisternal CSF, and upon the tissue concentrations of phosphocreatine and adenine nucleotides in anesthetized rats.* Acta physiol. scand. 1970. *78.* 433—447.

The effect of asphyxia upon the lactate, pyruvate and bicarbonate concentrations of brain tissue and cisternal CSF, and upon the tissue concentrations of phosphocreatine, ATP, ADP and AMP was studied in anesthetized and curarized rats, both at various times during (1, 2, 3 and 4 min) respiratory arrest, and after (1, 2, 5, 10 and 60 min) an arrest of 3 min. After 3 min of asphyxia the intracellular lactate concentration had increased to about 22 mMol/kg of i.c. water, while the calculated pH_i' had decreased about 0.6 units to 6.50. At the same time almost all phosphoc[illegible] here were [illegible] was prol[illegible] fall in p[illegible] the calc[illegible] con[illegible] cess[illegible] nor[illegible] restarting the respirator. The three min period of asphyxia was associated with a rapid and marked lactacidosis in arterial blood which was normalized at 60 but not at 10 min. In the CSF, the lactacidosis developed gradually during several min, and the lactate concentration had not normalized until in the 60 min group.

It is well known that a restriction of the oxygen supply to the brain leads to a breakdown of energy-rich tissue phosphates, such as phosphocreatine and ATP, and to an accelerated glucolysis with an accumulation of lactic acid. Most of the results on this

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subject have been obtained in situations of tissue hypoxia, induced by the administr tion of low oxygen concentrations (Gurdjian *et al* 1944, Thorn *et al* 1958, Schmal *et al* 1966), or by a complete interruption of the circulation to the brain (Thorn *al* 1958, Lowry *et al* 1964, Schmahl *et al* 1965) Although these studies have de scribed the general pattern of changes in the energy metabolism of the brain i hypoxia (see especially Lowry *et al* 1964), they provide a less useful basis for a appreciation of the tissue changes in clinically important conditions such as asphyxi or stagnant hypoxia This is partly due to the experimental models used but th most important shortcomings in our knowledge of hypoxic tissue events concern th rate and degree of restitution of the tissue changes after reoxygenation of the tissu the accompanying extracellular changes, and the relation between the changes in th energy metabolism and the corresponding alterations in the acid base parameters o the extra- and intracellular spaces Thus, recent results have clearly shown that bot the lung ventilation, and perhaps also the cerebral circulation may be critically in fluenced by the pH of cerebral extracellular fluids (Fencl *et al* 1966 for reference regarding cerebral blood flow, see symposium edited by Ingvar *et al* 1968) Such results make it pertinent to investigate the degree and duration of the acidosis which accompanies the changes in the energy metabolism in cases where the energy suppl to the cells has been inadequate (see Siesjo *et al* 1968 Kaasik *et al* 1968)

In the present report experiments are described in which extracellular concentra tions of lactate pyruvate and bicarbonate have been measured together with relevant tissue parameters such as lactate pyruvate, bicarbonate ATP, ADP, AMP and phosphocreatine during and after varying periods of asphyxia induced by respiratory arrest In order to minimize the drawbacks of depressive anesthetics the rats were anesthetized with nitrous oxide in oxygen Optimal procedures were used for freezing and extracting the tissue and blood gas tensions and pH were carefully controlled In a following paper cellular and extracellular changes will be reported during and after arterial hypotension caused by bleeding (Kaasik Nilsson and Siesjo 1969)

Methods

The experiments were performed on male rats of the Wistar strain weighing 300—400 g Anesthesia was induced with divinyl ether in a closed box, and maintained with 70 % nitrous oxide and 30 % oxygen The animals were immobilized with d tubocurarine i p and ventilated artificially with a Starling type respirator (Palmer Miniature Respirator) A catheter was introduced into the lower abdominal aorta through one femoral artery and used for sampling of arterial blood and for measurements of the blood pressure with an electromanometer (Elema Stockholm) A skin incision was made over the cranial vault and a plastic funnel was fitted into the incision for subsequent freezing of the tissue The skin was sutured around the funnel preventing any leakage of liquid nitrogen during the freezing The atlanto-occipital membrane was exposed by means of a longitudinal incision for subsequent sampling of 50—80 µl of CSF prior to the freezing of the brain Arterial blood was analysed for pO_2 pCO_2 and pH using microelectrodes (Eschweiler Kiel and Radiometer Copenhagen), and the haemo [illegible] ing a Vitatron Hb-meter In many ex [illegible] or subsequent analyses of [illegible] for bicarbonate in a few [illegible] ontent and for lactate [illegible] e to previous papers for details of measurements of CSF and tissue bicarbonate (Siesjo 196[illegible] Pontén and Siesjo 1964

Pontén 1966), and of CSF and tissue metabolites with enzymatic techniques (Hohorst *et al* 1959, Schmahl *et al* 1965, Lowry *et al* 1964, Granholm *et al* 1968)

Due to the low AMP concentrations obtained with the present freezing and extraction techniques, and to a variable contamination of the NADH with AMP (see Lowry *et al* 1964) the values obtained in the control group, and in the restitution groups, are only provisional. Thus, when corrections were made for the AMP concentration in blank solutions the net Δ F values for the brains analysed from the above groups sometimes were below 0 01. Accordingly, the AMP values in *e g* the control group varied between 0 and 0 14 mMoles/kg. It is clear that accurate measurements of the AMP concentrations of normoxic brains require more sensitive methods than the spectrofotometric one.

All tissue samples were extracted with perchloric acid at −15° C. All CSF, blood and tissue extracts were individually neutralized to pH 5 5—6 0 under electrometric pH control.

Asphyxia was induced for 1, 2, 3 or 4 min by stopping the respirator, and restitution after 3 min of asphyxia *was studied for periods of 1, 2, 5, 10 and 60 min after the restarting of the* respirator. In those animals which were analysed during the asphyxia, control arterial samples were taken before the induction of asphyxia, and at the end of the asphyctic period just before the sampling of CSF and the freezing of the brain tissue. In those rat groups in which restitution was studied, a third arterial sample was taken prior to sampling of CSF and brain tissue. In a few animals the EEG was recorded with bipolar leads, using brass screws secured into the skull bone.

For the calculation of intracellular bicarbonate, lactate and pyruvate, and of intracellular pH (pH_i', see Kjällquist *et al* 1969), certain assumptions had to be made. Thus, the tissue bicarbonate concentration was calculated from the total CO_2 content by subtracting the amount of physically dissolved CO_2. The latter was calculated by multiplying the solubility factor 0 0292 *mMol/kg/mm Hg (Siesjö 1962) with the tissue CO_2 tension, calculated from the arterial* CO_2 tension as described earlier (Pontén and Siesjö 1966). This procedure will include any error resulting from the nonsteady state situations encountered during brief periods of asphyxia. The bicarbonate, lactate and pyruvate concentrations in the intracellular phase were calculated by subtracting the amounts contained in the blood of the tissue (3 %), and in the extracellular space, the volume of which was assumed to be 12 % of the tissue weight (Rall *et al* 1962, [illegible]

[illegible] en by Pon- [illegible] 0 78 g/g of [illegible] tissue) was [illegible] 0 78/0 61, [illegible] r using the [illegible] (1969) for [illegible] red in the present work, *i e* maximally 4 min. In such short times the tissue buffer capacity should be predominantly determined by physico-chemical buffering (see Kjällquist *et al* 1969).

The bicarbonate concentration in the CSF, which was used to calculate the intracellular bicarbonate concentration, was not regularly measured due to the small CSF samples recovered. In those *cases it was assumed that the CSF bicarbonate concentration was decreased proportionally* to the increase in the CSF lactate concentration. This procedure was validated in the present [illegible]

[illegible] pO_2 was below 75 mm Hg and in two others, the [illegible] since there was a risk that the results might be [illegible] were excluded from the material.

Results

Arterial blood

In the course of the experiments measurements of pCO_2, pH and pO_2 were made in arterial blood before during and after the asphyxia, and in many experiments the lactate and pyruvate concentrations were also measured. Table I shows the values measured under control conditions as well as after 3 min of asphyxia, which was the standard period used for studying restitution. The values for 60 min of restitution

TABLE I Acid base and lactate/pyruvate values in arterial blood in control conditions, after 3 min of asphyxia, and after a restitution period of 60 min. Means ± SEM Number of experiments within parenthesis

Exp Group	Hb g %	pO_2 mm Hg	pCO_2 mm Hg	pH	Act HCO_3^- meq/l	Base excess meq/l	Lact mmoles/l	Pyr mmoles/l	La/Py
Control	15.2	124	37.2	7.399	22.1	−1.6	2.84	0.185	15.5
	(55)	(56)	(56)	(56)	(56)	(56)	(9)	(9)	(9)
Normoxia	±0.2	±4	±0.3	±0.003	±0.2	±0.2	±0.31	±0.019	±1.1
Asphyxia		14.2	77.7	7.137	24.0	−7.6	8.45	0.167	54
		(18)	(22)	(24)	(19)	(19)	(8)	(8)	(8)
3 min		±0.8	±1.5	±0.112	±0.5	±0.4	±1.58	±0.04	±6.0
Revival		102	37.2	7.428	23.6	−0.8	2.51	0.164	15.2
		(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
60 min		±7	±1.2	±0.020	±0.9	±1.1	±0.43	±0.024	±0.8

were also included in the table The table shows that three min of asphyxia was accompanied by a decrease of the pO_2 to about 14 mm Hg, and by an increase in the pCO_2 to about 78 mm Hg There was a good correspondence between the increase in the negative base excess and the increase in lactate Sixty min after re starting the respirator, all blood values, *including the lactate/pyruvate ratio*, had normalized

The changes in pO_2, pCO_2 pH base excess and lactate during and after asphyxia are shown in Fig 1 The figure shows that the pO_2 had decreased to values below 20 mm Hg already after 2 min and that it increased to values around 100 mm Hg already 1 min after the restarting of the respirator The changes in pCO_2 were slightly slower but not as slow as those in lactate Thus there was a significant accumulation of lactate and a decrease in base excess, in the blood 5 and 10 min after the asphyxia There was a good correspondence between the increase in lactate and the increase in the negative base excess during asphyxia but a relatively larger decrease in base excess in the 5 and 10 min groups after the asphyxia (see Discussion) It was apparent that the largest lactate accumulation and the lowest base excess values were obtained 1 min after the restarting of the respirator when the arterial pO_2 had already returned to values exceeding 100 mm Hg

Brain tissue

Table II gives the mean values for all directly measured metabolites in brain and CSF for the asphyctic and postasphyctic periods, together with the arterial CO_2 tensions for all experiments in which the tissue CO_2 content was measured As can be seen in the table, there were large changes in the lactate and pyruvate concentrations, in the phosphorylated compounds of the tissue and in the total CO_2 content

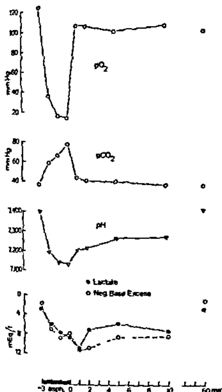


Fig 1

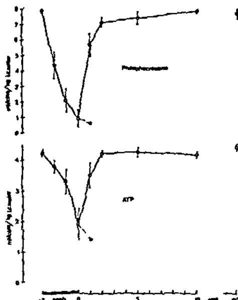


Fig 2

Fig 1 Changes in the arterial pO_2 pCO_2 pH (negative) base excess and lactate during a 3 min period of respiratory arrest in rats (black bar along time scale) as well as after restarting the respirator (time scale 0—60 min) The values given are the mean values from 4—6 individual rats

Fig 2 The phosphocreatine and ATP concentrations in rat brain tissue during and after a 3 min period of respiratory arrest Means ± S E M The values have been calculated for the intracellular water phase (see Methods and Table II) Filled circles represent mean values for a group of rats exposed to 4 min of respiratory arrest

During the asphyxia the lactate concentration increased rapidly to peak values around 16 mMol/kg with a proportionally much larger increase in lactate than in pyruvate and thus a corresponding increase in the lactate/pyruvate ratios (see below) The decrease in phosphocreatine was apparent already 1 min after the start of the asphyxia, and virtually all phosphocreatine had disappeared after 3 min of asphyxia At this time less than half of the ATP content remained and there were striking increases in the ADP and especially in the AMP concentrations It is seen that no appreciable further changes in the concentrations of the metabolites were seen when the asphyxia was prolonged to 4 min and it should be especially stressed that no further significant increase occured in the lactate concentration

TABLE II Acid base parameters and metabolites in brain tissue and cisternal CSF of rats und control conditions, during asphyxia, and at various times after a 3 min period of : phyxia The values are the means (± SEM) from measurements in 4—7 animals PaC in mm Hg, on other values in mmoles/kg

Exp group	$paCO_2$	Brain tissue TCO_2	PCr	ATP	ADP	AMP	La	Py	CSF La	Py
Control	37 2	13 71	5 05	2 72	0 40	0 08	1 46	0 090	2 78	0 179
	± 0 3	± 0 36	± 0 07	± 0 04	± 0 01	± 0 01	± 0 08	± 0 005	± 0 14	± 0 00
Asphyxia										
1 min	58 9	13 70	2 82	2 44	0 43	0 28	3 83	0 088	3 36	0 237
	± 3 4	± 0 65	± 0 53	± 0 13	± 0 03	± 0 04	± 1 19	± 0 007	± 0 43	± 0 01
2 min	66 1	11 70	1 34	2 13	0 68	0 33	10 25	0 100	3 48	0 199
	± 2 8	± 1 15	± 0 48	± 0 23	± 0 12	± 0 08	± 2 26	± 0 007	± 0 34	± 0 07
3 min	81 3	10 74	0 60	1 23	0 72	0 89	15 30	0 100	3 56	0 160
	± 4 1	± 0 55	± 0 35	± 0 32	± 0 10	± 0 25	± 1 96	± 0 010	± 0 18	± 0 01
4 min	76 3	9 91	0 39	0 91	0 68	0 89	16 48	0 078	4 19	0 176
	± 2 2	± 0 24	± 0 20	± 0 34	± 0 08	± 0 17	± 0 80	± 0 022	± 0 63	± 0 00
Restitution										
1 min	44 3	8 73	3 62	2 24	0 44	0 20	13 86	0 208	5 75	0 289
	± 4 7	± 0 45	± 0 50	± 0 22	± 0 13	± 0 04	± 3 23	± 0 032	± 0 17	± 0 07
2 min	41 5	9 69	4 57	2 67	0 33	0 10	7 17	0 231	4 62	0 217
	± 2 4	± 0 87	± 0 20	± 0 06	± 0 03	± 0 04	± 1 9	± 0 04	± 0 4	± 0 02
5 min	42 9	11 64	4 72	2 72	0 38	0 23	4 26	0 159	4 91	0 203
	± 2 7	± 0 82	± 0 25	± 0 09	± 0 02	± 0 03	± 1 5	± 0 03	± 0 44	± 0 01
10 min	39 6	12 35	4 99	2 66	0 37	0 15	2 17	0 106	4 15	0 195
	± 2 2	± 0 59	± 0 09	± 0 06	± 0 02	± 0 02	± 0 42	± 0 014	± 0 27	± 0 013
60 min	37 2	13 57	4 86	2 81	0 32	0 09	1 53	0 090	2 98	0 194
	1 2	± 0 75	± 0 19	± 0 04	± 0 02	± 0 04	± 0 09	± 0 006	± 0 20	± 0 006

There were quite clearcut differences in the rate and degree of normalization of the tissue metabolites during restitution Thus control values were approached or reached for ADP after 1 min for ATP and AMP after 2 min for phosphocreatine after 5 min, and for lactate and pyruvate after 10 min The changes in the phosphocreatine and ATP concentrations during and after the asphyxia have been illustrated in Fig 2, but the concentrations were calculated for the intracellular water phase to facilitate comparisons with intracellular pH_i' changes (see below) It can be seen in the figure that the three min asphyxia was accompanied by a decrease in the ATP concentration of about 2 mmoles/kg whereas the phosphocreatine concentration fell by about 7 mmoles/kg of i c water

TABLE III Comparison between the increase in the lactate concentrations and the decrease in the bicarbonate concentrations in cisternal CSF during brain hypoxia (see text)

Exp group	$paCO_2$ mm Hg	HCO_3 meq/l	Lactate meq/l	Δ HCO_3 meq/l	Δ Lactate meq/l
Control	36 7 (4) ± 0 6	27 83 ± 0 27	2 78 ± 0 14		
Asphyxia 3 min	70 9 (4) ± 2 8	27 43 ± 0 46	3 56 ± 0 18	0 4	0 8
"Bleeding	15 9 (4) ± 2 2	24.29 ± 0 57	6 27 ± 0 42	3 5	3 5

CSF

In the calculation of the intracellular bicarbonate concentrations it was assumed that the CSF bicarbonate concentration was decreased proportionally to the increase in the lactate concentration This assumption was tested in 4 rats which were exposed to 3 min of asphyxia and analysed for the CSF bicarbonate concentration. However since there were very small increases in the lactate concentration in the asphyxia groups (see Table II), an additional group of 4 rats was bled to give a mean blood pressure of 30—35 mm Hg for 10 min (see Kaasik *et al* 1969) before the sampling of CSF Table III compares the bicarbonate concentrations obtained in a control group of 4 rats with the bicarbonate concentrations in the 3 min asphyxia group and the 10 min "bleeding group as well as the lactate concentrations in the control asphyctic and bleeding groups (see Table II and Kaasik *et al* 1969) The results show that there was a good agreement between the decrease in the CSF bicarbonate and the increase in the lactate concentration and the assumption of a one to-one relationship thus seems justified

The highest lactate values obtained were seen after the resumption of normal breathing and the lactate values as well as the lactate/pyruvate ratios were still appreciably elevated 10 min after the restarting of the pump These changes reflect the lag in the lactate and pyruvate diffusion from the tissue to the CSF and also the slow clearing of the CSF lactate (see Discussion)

Calculated intracellular parameters

The true cellular changes in lactate pyruvate and bicarbonate are more apparent if corrections are made for the extracellular concentrations. Corrections were therefore applied for the blood and CSF concentrations using tissue blood and ECF volumes of 3 and 12 % respectively (see Methods However in order to estimate the decrease in bicarbonate caused by the accumulation of nonvolatile acids and thus not due to the increased CO tensions base excess values were tentatively calculated as described in the methods

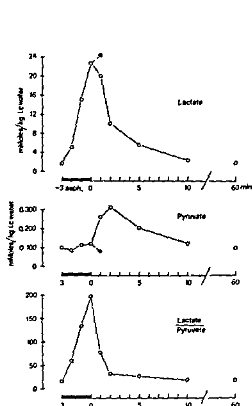


Fig 3

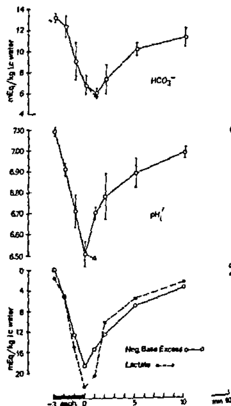


Fig 4

Fig 3 Intracellular lactate and pyruvate concentrations, and lactate/pyruvate ratios of rat brains during and after a 3 min period of respiratory arrest The intracellular concentrations were calculated from the values given in Table II after correction for the amounts contained in the blood and CSF volumes of the tissue (see Methods) Filled circles represent mean concentrations in a group of rats exposed to 4 min of asphyxia (lactate/pyruvate ratio of 300 not indicated)

Fig 4 Intracellular bicarbonate pH, and (negative) base excess of rat brains during and ... and the pH were ... volumes of the ... racellular phase ... lar lactate concentrations (filled circles) Triangles denote the mean values for HCO_3 and pH_i in a group exposed to respiratory arrest for 4 min

Fig 3 shows the changes in the intracellular lactate and pyruvate concentration and in the lactate/pyruvate ratio There was an increase in the intracellular lactate concentration of about 20 mmoles/kg of i c water during the three min asphyxia, but a very small if any further increase if the asphyxia was prolonged to 4 min The pyruvate concentration remained unchanged during the asphyxia but increased markedly when the animals were reoxygenated i e at a time when the lactate concentration was falling rapidly (see Discussion) These relations were reflected in the lactate/pyruvate ratio, which fell as rapidly during the restitution phase as it in-

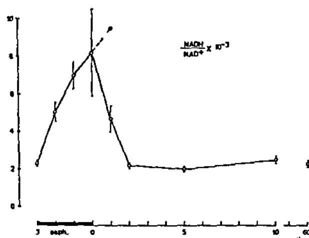


Fig. 5. Intracellular (cytoplasmatic) NADH/NAD ratios in the rat brain during and after respiratory arrest for 3 min (mean value for 4 min group denoted by filled circle). Means ± S.E.M. The NADH/NAD ratios were calculated from the intracellular lactate and pyruvate concentrations and from the pH values assuming a K value of $1.11 \cdot 10^{-11}$ (see Methods).

creased during the asphyctic phase. There was a considerable retention of lactate and pyruvate 2 and 5 min after the restarting of the pump but not at 10 min since the slightly increased mean lactate concentration at that time was due to a single high value (3.74 mmoles/kg of wet tissue).

Fig. 4 shows the calculated intracellular bicarbonte concentrations, the calculated pH′ values and the calculated base excess values which have been compared to the intracellular lactate values. It can be seen that at 3 and 4 min of asphyxia, when the intracellular lactate had increased to 20—25 mmoles/kg of i.c. water, the intracellular bicarbonate was still 7—8 mmoles/kg. The pH_i' fell almost linearly during the asphyxia from 7.09 in the control group to 6.50 in the 3 min group. In the 4 min group there was only a slight further fall. During restitution there was a gradual return of pH′ towards control values and the 10 min group still showed an appreciable intracellular acidosis.

The calculated base excess values showed changes which were in fair agreement with the intracellular lactate concentrations but the 2, 5 and 10 min restitution groups indicated that the intracellular space remained more acid than corresponded to the lactate retained (see Discussion).

With a knowledge of pH′ and of the lactate and pyruvate concentrations, the intracellular NADH/NAD ratio could be calculated according to the equation

$$\frac{NADH}{NAD^+} = \frac{Lact}{Pyr} \cdot \frac{K}{H^+}$$

setting K equal to $1.11 \cdot 10^{-11}$ (Williamson *et al.* 1967, see also Granholm and Siesjö 1969). Fig. 5 shows that the calculated NADH/NAD ratio increased about 4-fold during the 3—4 min of asphyxia and that the ratio quickly returned to control values which were reached already 2 min after restarting the respirator.

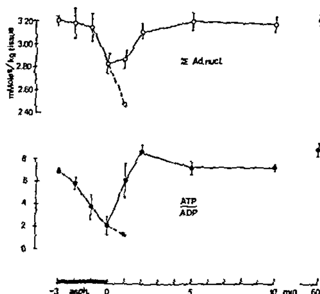


Fig 6 The sum of the adenine nucleotides and the ATP/ADP ratios of rat brains during and after respiratory arrest for 3 min (mean values for 4 min group indicated by interrupted lines) Means±S E M

Adenine nucleotides

It has already been shown (see Table II) that the concentrations of ATP, ADP, and AMP normalized already 2 min after the restitution of normal breathing However since the energetic potential of a tissue is often expressed as the ATP/ADP ratio (see critical discussion by Schmahl *et al* 1965) and since the sum of the adenine nucleotides has been shown to be rapidly changed in tissue hypoxia (see *e g* Lowry *et al* 1964) these two parameters have been given in Fig 6 The figure shows that the ATP/ADP ratio had normalized at 2 min but that the sum of the adenine nucleotides was not at control values until at 5 min

EEG

A few control experiments showed that an isoelectric EEG was obtained 2–3 min after the induction of asphyxia and that the EEG reappeared in the 1 min restitution group However a preliminary frequency analysis indicated that the EEG in the 10 min restitution group still contained more low frequency components than the control EEG

Discussion

The main aim of the present experiments was to study the acid base changes which must accompany the pronounced derangement of the oxidative metabolism in the brain during asphyxia However since the literature does not give any hints to the changes which occur in the concentrations of phosphocreatine and of the adenine nucleotides in asphyxia (*i e* during hypoxemia and hypercapnia with patent circulation), these changes have been described in detail together with the lactate and pyruvate concentrations in the tissue and in the CSF

The most complete study of changes affecting the oxidative metabolism after an interruption of the blood supply to the brain is that of Lowry *et al* (1964) but the technique used precluded measurements of the restitution of the tissue changes Scattered measurements have been reported of the restitution in lactate/pyruvate in phosphocreatine and in the adenine nucleotides after cerebral hypoxia induced by low oxygen mixtures (Gurdjian *et al* 1944 Schmahl *et al* 1966) These results have shown that the restitution of the phosphocreatine and ATP values is quicker than the corresponding restitution of the lactate and pyruvate concentrations The measurements performed were too few, however to give an adequate picture of the pattern of restitution nor were any CSF metabolites or any acid base parameters studied

Methods and calculations

Before discussing the implications of the present results a few methodological points must be brought up for discussion. Thus the results are based on analyses of tissue metabolites after freezing the tissue *in situ* The method used for freezing the tissue apparently gives a very good indication of the true *in vivo* state (see Discussion in Granholm *et al* 1968) but the time taken to freeze the tissue will undoubtedly introduce a discrepancy between the asphyxia times and the actual freezing times since it takes 40—60 sec for the brain to get solidly frozen However since the supratentorial parts were used for analyses it can be estimated that the concentrations measured represent the metabolic state approximately half a minut after the commencement of the freezing

The non steady state conditions prevailing during asphyxia and immediately after the resumption of a normal ventilation will introduce an uncertainty in the calculation of intra (and extra)cellular pH values Thus the calculation of tissue pCO values from the arterial CO tensions (Ponten and Siesjo 1966 are only directly applicable to steady state conditions and the errors introduced by the nonsteady state are difficult to calculate However even if the calculated tissue pCo should be in error by 10 mm Hg in *e g* the 3 min asphyxia group the error in the calculated pH is only 0 05—0 06 *i e* less than 10 % of the total pH change Moreover the pCO error is probably less than that since the CO equilibration between blood and cells will occur in seconds when the pCO change occurs in the blood (*i e* not in the inspired gas mixture) or in the tissue (see discussion by Siesjo 1968) It can be tentatively concluded that the errors introduced will probably be quite insignificant for the conclusions regarding the pH' changes

The errors introduced by inhomogeneities in the extracellular bicarbonate concentrations and by variations in the size of the extracellular compartment (see Methods) will probably also be of small significance When we correct for the ECF bicarbonate the concentrations in the CSF is multiplied by the size of the compartment. Thus if we assume that in the 3 min asphyxia group the mean ECF bicarbonate is overestimated by 5 meq/kg and the ECF volume by 50 % the calculated intracellular bicarbonate will be underestimated by about 2 meq/kg of intracellu

water, and pH_i' by about 0 1 pH units In spite of the fact that asphyxiation has been reported to decrease the extracellular volume (van Harreveld 1966), the above calculation probably represents an unrealistically large error, since a large part of the extracellular bicarbonate should be confined to the bulk CSF phases which are not likely to alter their volumes appreciably We can thus tentatively conclude that any error introduced in the calculations of pH_i' will probably not exceed 0 1 pH units and it may be appreciably lower than this figure

Blood and CSF changes

It deserves mentioning that the arterial pO_2 decreased to a mean value of below 40 mm Hg already after 1 min of asphyxia, and to values below 20 mm Hg after 2 min There occured only a small further decrease in pO_2 between the second and the fourth min, and no further increase in pCO_2 between the third and fourth min The absence of further increases in the pCO_2 was obviously due to a fall in the rate of oxidative decarboxylations when oxygen became unavailable Both the pO_2 and the pCO_2 approached or reached control values already 1 min after the resumption of ventilation and it should thus be borne in mind that the tissue must have had an adequate oxygen tension and a near normal pCO_2 in all restitution groups studied

There was a fair correspondence between the increase in the negative base excess of blood, and the increase in the lactate concentration during asphyxia However during the restitution phase the lactate seemed to normalize quicker than the base excess, suggesting that lactate was not eliminated with a stoichiometrical amount of hydrogen ions, or that some of the hydrogen ions added to the blood were not primarily associated with lactate anions

In the CSF there was a delayed increase in the lactate and a delayed fall towards normal values Thus 10 min after restarting the pump there was still an excess of lactate in the CSF (4 15 mmoles/kg) and an increased lactate/pyruvate ratio (21 3) These delayed CSF changes are explained by diffusion lags in the relatively stagnant CSF pools The lactacidosis of the CSF and the increased lactate/pyruvate ratio have their importance in indicating either that a hypoxic change in the tissue exists or that it has existed in the preceding period (cf Siesjo Kjallquist and Zwetnow 1968)

Tissue changes

The *lactate concentration* increased by about 14 mmoles/kg of wet tissue during the 3 min asphyxia This is a mean increase of about 4 6 mmoles per kg and min a figure which compares very well with that of 4 3 mmoles per kg and min reported by Schmahl *et al* (1965) However since the lactate is liberated in the water phase of the cells, the total concentration change should be about 21 mmoles/kg of ic water or 7 mmoles per kg and min This increase in lactate occurred without significant changes in the *pyruvate concentration* and was thus associated with a rapid rise in the lactate/pyruvate ratio The rapid decrease in the lactate concentration during the first 2 min of the restitution phase and the marked increase in the pyruvate con

centration, gave rise to a very rapid fall in the lactate/pyruvate ratio These changes are logically interpreted as a very fast reoxidation of cytoplasmic NADH and an equivalent and rapid shift in the lactate/pyruvate ratio Apparently, the flux of reducing equivalents across the mitochondrial membrane was very much faster than the corresponding transfer and oxidation of pyruvate the (cytoplasmic) concentration of which remained increased for 5—10 min

It has previously been reported that whereas an ischemic cycle in the liver is followed by a very fast restitution of the mitochondrial NADH/NAD ratio and in the ATP, ADP and AMP concentrations, the lactate/pyruvate ratio remained increased for several min (Chance *et al* 1965) These findings were interpreted as a slow reoxidation of the cytoplasmatic NADH system The present results show that, in the brain the lactate/pyruvate ratio normalized very quickly, and if allowance is made for the pH_i' changes, the cytoplasmatic NADH/NAD ratio appeared to normalize *pari passu* with the normalization of the ATP, ADP, and AMP concentrations (see below)

The drastic reduction of pH_i' during the asphyxia was of course due to both the increased CO_2 tension, and to the markedly increased lactic acid concentration The influence of lactic acid alone can be appreciated by considering the 1 min restitution group, in which the intracellular lactate was still about 20 mmoles/kg but in which the CO_2 tension apparently had approached control values It is striking that the accumulation of more than 20 mmoles of lactate per kg of i c water was associated with a decrease in the intracellular bicarbonate concentration of less than 10 meq/kg This is partly due to the carbon dioxide buffered by nonbicarbonate buffers (during apshyxia), and partly to the relatively high intracellular buffer capacity Thus, if the buffer equation described by Ponten (1966) is recalculated to intracellular concentrations (see Methods), a fair agreement was obtained between the calculated decrease in buffer base, and the increase in the intracellular lactate concentration

There is *a priori* no reason to assume that there should be a perfect stoichiometrical relationship between increase in lactate and decrease in buffer base Thus although lactic acid seems to be the only acid which is known to be liberated in appreciable amounts in acute tissue hypoxia there are other reactions which involve changes in the hydrogen ion concentrations These reactions include the liberation of protons when substrate hydrogen accumulates in the redox systems of the electron transport chain and when ATP is hydrolysed to ADP and inorganic phosphate but it has been pointed out that other reactions such as the hydrolysis of phosphocreatine may lead to a removal of hydrogen ions (Hill 1955 see discussion by Siesjo Kaasik Nilsson and Ponten 1968) When the ATP and phosphocreatine concentrations were recalculated to intracellular concentrations (Fig 2) it can be estimated that 3—4 min of asphyxia led to decreases in the ATP and phosphocreatine concentrations of 2 and 7 mmoles/kg of i c water respectively changes which may well influence the intracellular pH' Before the influence of such reactions on the intracellular hydrogen ion metabolism can be assessed however more precise information on intracellular buf systems must be obtained Until such information is available it should be r

that the intracellular phase appeared to remain acid in the 10 min restitution group in spite of the fact that the lactic acid had decreased towards control values

The decrease in the lactic acid production after 3 min was probably not due to lack of substrate (*i e* glucose) but may be due to a pH inhibition of glucolysis (*cf* Hill 1955) At this point the energy production must have been close to zero and it was also found that only about 50 % of the animals survived the asphyxia until the fourth min.

Appraisal of "tissue hypoxia"

It is of considerable interest to try to define the parameters which form the most sensitive indicators of the presence of an insufficient oxygen supply to the tissue Measurements of the oxygen supply itself probably represent a rather insensitive method, and it appears more logical to define the insufficient oxygen supply in terms of its influence on metabolites or redox systems in the tissue Of the various metabolites and redox systems which are easily accessible for measurements the lactate/pyruvate ratio the phosphocreatine, ATP and AMP concentrations and the ATP/ADP and the ATP/AMP ratios have been reported to indicate the degree of cellular hypoxia (see Bücher and Klingenberg 1958, Hohorst 1960 Huckabee 1960 Minard and Davis 1962 Schmahl *et al* 1965 Lowry *et al* 1964) In the present experiments the induction of asphyxia was very rapidly reflected in the lactate/pyruvate the ATP/ADP and the ATP/AMP ratios as well as in the phosphocreatine, lactate AMP and ATP concentrations In the restitution phase ATP, ADP and AMP as well as the ATP/ADP (and ATP/AMP) ratios normalized already after 2 min Since also the calculated cytoplasmatic NADH/NAD ratio had normalized at that time it seems logical to assume that the energy metabolism of the tissue had normalized However it should be borne in mind that neither the phosphocreatine concentration nor the sum of the adenine nucleotides appeared to have normalized at that time Thus even if the metabolic pattern at 2 min indicates the normalization of the mitochondrial and cytoplasmatic redox ratios rephosphorylation of ATP and phosphocreatine appeared incomplete and the tissue still showed a marked lactacidosis and an altered EEG pattern Thus when restitution of the tissue metabolism after hypoxia is discussed the parameters must evidently be critically defined

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The Effect of Arterial Hypotension upon the Lactate, Pyruvate and Bicarbonate Concentrations of Brain Tissue and Cisternal CSF, and upon the Tissue Concentrations of Phosphocreatine and Adenine Nucleotides in Anesthetized Rats

By

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Abstract

KAASIK, A. E., L. NILSSON and B. K. SIESJÖ. *The effect of arterial hypotension upon the lactate, pyruvate and bicarbonate concentrations of brain tissue and cisternal CSF, and upon the tissue concentrations of phosphocreatine and adenine nucleotides in anesthetized rats.* Acta physiol. scand. 1970. 78. 448—458.

The effect of stagnant hypoxia upon the lactate, pyruvate and bicarbonate concentrations of brain tissue and cisternal CSF, and upon the tissue concentrations of phosphocreatine, ATP, ADP, and AMP was studied in anesthetized and curarized rats. The stagnant hypoxia was induced by arterial bleeding to a mean arterial blood pressure of 25—35 mm Hg. The hypotension was found to lead to moderate decre [illegible] and to increases in the AMP and lactate [illegible] pressure was found to give a rapid restitut [illegible] restitution of the phosphocreatine concentration, and to a very slow disappearance of the [illegible] and pyruvate accumulated in the tissue and in the CSF. However, the cytoplasmatic NADH/NAD+ ratio calculated from the intracellular lactate and pyruvate concentrations and from the pH$_i$ values, was found to normalize already 2 min after the reinfusion of the blood. There was a linear relationship between the lactate accumulated in the intracellular space, and the pH$_i$', and the slope of the line (Δ lactate/Δ pH) indicated that the intracellular buffer capacity thus calculated, is lower than that of whole blood *in vitro*.

In a previous publication we described the effect of asphyxia, induced by respiratory arrest, on the lactate, pyruvate and bicarbonate concentrations of rat brain tissue and cisternal CSF, as well as on the phosphocreatine, ATP, ADP and AMP concentra-

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tions of the tissue In the present paper we will describe changes in the same tissue and CSF parameters accompanying stagnant hypoxia induced by arterial bleeding This type of tissue hypoxia differs from that caused by respiratory arrest in that it is accompanied by a decreased tissue blood flow and by arterial hypocapnia at an unchanged arterial oxygen tension In order to standardize the stagnant hypoxia at a level which would by certainty lower the cerebral blood flow (Haggendal 1965 Harper 1965), the animals were bled to a mean arterial blood pressure of 25—35 mm Hg The blood pressure was upheld at this value for 3—10 min Groups of animals were then reinfused with blood after a standardized hypotensive period of 5 min and studied after an additional 2—60 min period It will be shown that although a five min period of low blood pressure was accompanied by tissue changes which were less marked than the corresponding changes after 3 min of asphyxia the hypotension led to relatively longlasting metabolic changes indicative of a more serious derangement of the oxidative metabolism of the tissue

Methods

The experiments were performed on rats of the Wistar strain, weighing 300—450 g The
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ADP, AMP [illegible] was calculated
from the CO_2 tension and from the bicarbonate concentration after correction for the bicarbonate contained in the blood and CSF volumes of the tissue (assumed to be 3 and 12 %
respectively) However, since a de
the present degree, the tissue C
cisternal CSF in control experim
decrease in the CSF bicarbonate
All animals were kept normoth
mittent heating The blood pressure was measured in a cannula inserted into the abdominal aorta from one femoral artery, using an electromanometer (Elema, Stockholm) the mean
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TABLE I The effect of arterial hypotension on acid base parameters and on lactate and pyruvate concentrations in arterial blood Means ± S E M Number of experiments within parentheses The control values for lactate and pyruvate were taken from a previous publication (Kaasik et al 1969)

Exp group	Hb g %	pO_2 mm Hg	pCO_2 mm Hg	pH	Act HCO_3 meq/l	Base Excess meq/l	Lactate mmoles/l	Pyruvate mmoles/l
Control	15 2 (5) ± 0 34	97 0 (5) ± 2 3	39 1 (5) ± 0 7	7 373 (5) ± 0 003	21 9 (5) ± 0 3	— 2 3 (5) ± 0 2	2 84 (9) ± 0 31	0 185 (9) ± 0 019
3 min		98 0 (5) ± 5	29 2 (5) ± 3 0	7 318 (5) ± 0 061	14 0 (5) ± 0 7	— 10 5 (5) ± 1 7	8 66 (5) ± 1 18	0 152 (5) ± 0 013
Control	13 9 (6) ± 0 6	134 (7) ± 7	37 6 (7) ± 0 8	7 377 (7) ± 0 004	21 4 (7) ± 0 4	— 2 6 (7) ± 0 4	2 84 (9)	0 185 (9)
5 min	12 7 (7) ± 0 3	121 (5) ± 6	19 6 (6) ± 2 0	7 315 (5) ± 0 042	9 6 (6) ± 0 6	— 14 9 (5) ± 0 9	12 56 (6) ± 1 45	0 205 (6) ± 0 028
Control	16 0 (4) ± 0 3	119 (4) ± 14	38 2 (4) ± 1 1	7 383 (4) ± 0 005	22 1 (4) ± 0 4	— 1 8 (4) ± 0 1	2 84 (9)	0 185 (9)
10 min		103 (4) ± 5	18 7 (4) ± 3 3	7 269 (4) ± 0 047	8 0 (4) ± 1 0	— 17 4 (4) ± 1 5	13 03 (4) ± 0 69	0 137 (4) ± 0 0[illegible]

Results

Arterial blood

The acid base parameters of arterial blood are shown in Table I and in Fig 1 Table I compares the values measured after 3 5 and 10 min of arterial hypotension with the control values obtained in the individual groups prior to the withdrawal of blood There were no significant changes in the arterial pO_2 in any of the hypotensive or recovery groups and the tissue changes (see below) thus occurred in animals which all had pO_2 values exceeding 90 mm Hg and Hb concentrations exceeding 10 mg % The acid base changes were characterized by moderate decreases in the pH but by marked decreases in the base excess values and by substantial incrases in the lactate concentrations The absence of marked pH changes was due to a progressive decrease in the arterial CO_2 tensions during the hypotension which occurred in spite of a *constant ventilation and a constant rectal temperature* In the 5 and 10 min groups arterial CO_2 tensions as low as 11—13 mm Hg were recorded but the scatter of the values showed that the hypotension led to a variable depression of the CO_2 output from the tissues

Fig 1 illustrates the changes in the pCO_2 pH base excess and lactate values during and after a 5 min hypotensive period The figure shows that the arterial CO_2 tension returned to control values already in the 2 min group The rapid increase in the CO_2 tension at that time gave rise to a second fall in pH to about 7 2 and the

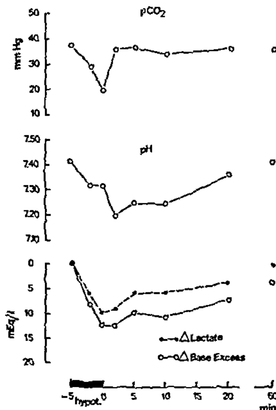


Fig 1 Arterial pCO_2, pH, lactate concentration and (negative) base excess during (black bar at time −5 to 0 min) and after (time 0 to 60 min) an arterial hypotension of 25—35 mm Hg induced by arterial bleeding Note marked decrease in the arterial CO_2 tension as well as discrepancy between the lactate and base excess values

pH did not return to control values until in the 60 min group The low pH values were apparently partly due to the longlasting increase in the lactate concentration, but the fact that the increase in the negative base excess exceeded the increase in the lactate concentration both during and after the 5 min hypotensive period indicates that also other factors were responsible for the blood acidosis (see Discussion)

Brain tissue

Table II lists all the directly measured parameters in brain tissue and CSF together with the arterial CO_2 tensions The tissue CO_2 tensions in the hypotensive period was obtained from the CO_2 tensions measured directly in cisternal CSF in the 5 and 10 min hypotensive groups but were calculated from the arterial CO_2 tensions in the restitution groups (see Fig 2 below) The CSF CO_2 tension (see Ponten and Siesjo 1966 Brzezinski *et al* 1967) was measured in 11 rats which were bled to a mean arterial blood pressure of 25—35 mm Hg Fig 2 shows that 5 (unfilled circles) and 10 (filled circles) min of hypotension led to a marked increase in the CSF/arterial pCO_2 difference Since there were no significant differences between the 5 and 10 min groups the tissue CO_2 tension for all rats in the 3 5 and 10 min hypotensive groups was derived from the line drawn in Fig 2

TABLE II The arterial CO_2 tension, the brain tissue concentrations of total CO_2, lactate, pyruvate phosphocreatine and adenine nucleotides, as well as the lactate and pyruvate concentrations in cisternal CSF, of rats bled to a mean arterial blood pressure of 25—35 mm Hg Means ± SEM of 4—7 rats The control values were taken from the previous communication (Kaasik et al 1969) $PaCO_2$ in mm Hg all other values in mmoles kg

		Brain Tissue							CSF	
Exp group	$PaCO_2$	TCO_2	PCr	ATP	ADP	AMP	La	Py	La	Py
Control	37 2 ± 0 4	13 71 ± 0 36	5 05 ± 0 07	2 72 ± 0 04	0 40 ± 0 01	0 08 ± 0 01	1 46 ± 0 08	0 030 ± 0 005	2 78 ± 0 14	0 178 ± 0 009
Bleeding										
3 min	29 2 ± 3 0	10 34 ± 1 04	4 59 ± 0 06	2 63 ± 0 06	0 29 ± 0 06	0 11 ± 0 01	5 49 ± 1 17	0 169 ± 0 028	4 56 ± 0 45	0 186 ± 0 003
5 min	19 6 ± 2 0	8 19 ± 0 67	3 16 ± 0 68	2 37 ± 0 17	0 49 ± 0 06	0 21 ± 0 06	10 18 ± 2 13	0 193 ± 0 019	5 53 ± 0 72	0 199 ± 0 018
10 min	18 7 ± 3 3	7 41 ± 0 78	2 97 ± 1 01	1 98 ± 0 50	0 46 ± 0 08	0 65 ± 0 43	11 13 ± 3 95	0 161 ± 0 037	6 27 ± 0 42	0 198 ± 0 013
Restitution										
2 min	36 1 ± 2 7	8 47 ± 1 00	4 58 ± 0 30	2 74 ± 0 10	0 31 ± 0 03	0 12 ± 0 03	8 12 ± 1 96	0 231 ± 0 018	7 82 ± 0 36	0.288 ± 0 020
5 min	36 5 ± 2 2	10 28 ± 1 46	4 44 ± 0 37	2 72 ± 0 09	0 32 ± 0 04	0 10 ± 0 03	6 57 ± 2 72	0 205 ± 0 054	6 84 ± 1 53	0 255 ± 0 016
10 min	34 2 ± 2 2	11 65 ± 0 41	4 41 ± 0 30	2 73 ± 0 07	0 34 ± 0 02	0 11 ± 0 03	4 14 ± 1 25	0 145 ± 0 010	6 26 ± 0 86	0 240 ± 0 015
20 min	36 4 ± 0 5	11 83 ± 0 62	4 73 ± 0 30	2 65 ± 0 09	0 35 ± 0 02	0 10 ± 0 02	3 28 ± 0 66	0 147 ± 0 016	5 66 ± 0 67	0 265 ± [illegible]
60 min	36 3 ± 0 9	13 62 ± 0 29	5 14 ± 0 13	2 98 ± 0 05	0 40 ± 0 01	0 03 ± 0 01	1 67 ± 0 09	0 096 ± 0 005	3 14 ± 0 19	0 197 ± 0 010

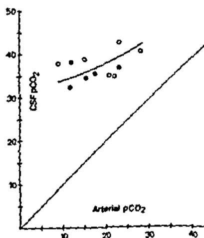


Fig 2 The relation between the arterial CO_2 tension and the CO_2 tension in cisternal CSF of rats exposed to a lowering of the mean arterial blood pressure to 25—35 mm Hg for 5 (unfilled circles) or 10 (filled circles) min The straight oblique line is the line of equality The curve, which was drawn by inspection was used to derive the mean tissue CO_2 tension in the individual experiments

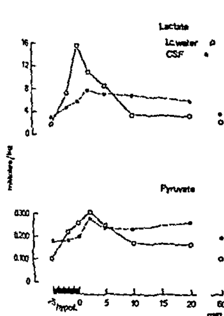


Fig 3

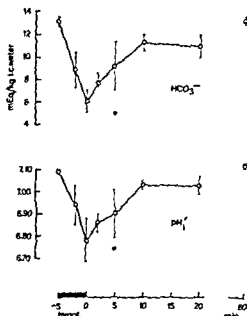


Fig 4

Fig 3 The lactate [illegible] ular spaces in the rat bra[illegible] concentrations were equ[illegible] concentrations were calc[illegible] the figures given in Table II after correction for the amount of lactate and pyruvate of the blood and the extracellular volumes, respectively (see Methods

Fig 4 [illegible] of the ra[illegible] for th[illegible] eans ± [illegible]

The hypotension led to clearcut changes in the concentrations of the tissue metabolites (Table II) Thus there were highly significant decreases in the phosphocreatine and ATP concentrations at 5 min, which were even more marked at 10 min. There were similar but inverse changes in the AMP concentrations while the ADP concentration varied only little There were marked increases in the tissue lactate and pyruvate concentrations, and a marked decrease in the total CO_2 content, but these changes are better visualized if the corresponding intracellular parameters are considered (see below)

During restitution after a 5 min period of hypotension the ATP and AMP approached or reached control values already within 2 min while there were significant decreases in the phosphocreatine concentration in the 2, 5, and 10 min groups In the same groups the ADP concentrations were decreased In the 60 min group all values, including the lactate and pyruvate concentrations, had normalized

Intra- and extracellular changes

The intracellular lactate, pyruvate and bicarbonate concentrations were

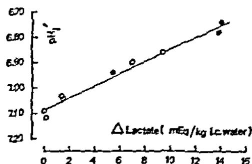


Fig 5

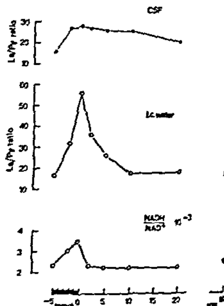


Fig. 6

Fig 5 The relation between the increase in the intracellular lactate concentration and the calculated pH_i, in the brain of rats during (filled circles) and after (unfilled circles) an arterial hypotension (see Table II, Fig 3, and text) The unfilled circle with zero Δ lactate denotes control group The slope of the straight line, drawn by inspection through the points, gives a buffer capacity ($\beta = \Delta B/\Delta$ pH) of 0 042

Fig. 6 The lactate/pyruvate ratios of cisternal CSF and of brain intracellular water as well as the calculated cytoplasmatic $NADH/NAD^+$ ratio in rats during and after an arterial hypotension (cf Fig. 1 Note normal $NADH/NAD^+$ ratio in all restitution groups in spite of high CSF and intracellular lactate/pyruvate ratios

after correcting for the corresponding amounts confined to the blood and the extracellular volumes of the tissue assumed to be 3 and 12 % respectively, of the tissue weight (see Kaasik Nilsson and Siesjö 1969) Fig 3 illustrates the lactate and pyruvate concentrations of the intra- and extracellular spaces The figure shows the rapid increase and the slow disappearance of the intracellular lactate as well as the delayed changes in the CSF lactate and pyruvate concentrations Thus, during and immediately after the hypotensive period there was a disparity between the increases in the intra and extracellular concentrations in that the intracellular lactate and pyruvate concentrations greatly exceeded the corresponding CSF concentrations but in the 10 and 20 min restitution groups the increases in the lactate concentrations were about equal in the two compartments The increase in the intracellular pyruvate concentration in the 2 min restitution group which occurred in spite of a falling lactate concentration was related to the fast reoxidation of the cytoplasmatic NADH/NAD system (see below and Kaasik et al 1969

Fig 4 shows the bicarbonate concentration and the calculated pH' values of the intracellular space The intracellular bicarbonate concentration fell to about 6 5 meq/kg of i.c. water in 5 min 5 5 meq/kg in the 10 min hypotensive group), and

this decrease in bicarbonate was associated with a fall in pH_i' from 7 1 to 6 8 (6 75 in the 10 min group) Since there were rather small variations in the tissue CO_2 tensions between the groups the decrease in pH_i' should be primarily due to the accumulation of lactic acid (see Discussion) Fig 5 shows that the relation between the increase in the intracellular lactate and the pH_i' was linear, and that no significant differences were found between the hypotensive and the restitution groups

With a knowledge of the intracellular lactate and pyruvate concentrations and the pH_i' values, the cytoplasmatic NADH/NAD ratios could be calculated (see Kaasik *et al* 1969) Fig 6 illustrates the lactate/pyruvate ratios in CSF and in the intracellular space, as well as the NADH/NAD ratios The figure shows that whereas the lactate/pyruvate ratios were elevated in the 2, 5, 10 and 20 min groups the NADH/NAD ratio was normalized already in the 2 min group

Discussion

It is well known that if the mean arterial blood pressure is reduced to such a degree that the cerebral circulation is diminished, there are changes in the tissue metabolism suggestive of a gross interference with the oxygenation of the tissue These changes include the breakdown of phosphocreatine and ATP and the accumulation of AMP and lactate (LePage 1946, Reulen *et al* 1968) The tissue seems to have a good capacity of resisting decreases in the perfusion pressure in that changes in the cerebral circulation and in tissue metabolites, occur first when the mean arterial blood pressure is reduced to below 40—60 mm Hg (Haggendal 1965 Harper 1965 Reulen *et al* 1968 see also Haggendal *et al* 1969) There seems to be a narrow margin between the beginning of the circulatory and metabolic changes and the occurrence of an irreversible structural tissue damage, which is reported to occur below a perfusion pressure of 25 mm Hg (Brierley *et al* 1969) When such irreversible changes first occur, they seem to affect certain cortical and subcortical grey matter areas which are situated in the boundary zones between the main distribution fields of major cerebral arteries (see Brierley *et al* 1969)

The aim of the present study was to quantitate changes occurring in the tissue acid base and energy metabolism during and after a decrease in the mean arterial blood pressure which was sufficiently marked to give a reduction of the cerebral blood flow, but not as marked so as to lead to a permanent structural damage It was also the intention to compare the changes observed during and after the arterial hypotension with those measured during and after asphyxia induced by respiratory arrest, as described in a preceding communication (Kaasik *et al* 1969)

The present arterial hypotension, like the previously studied asphyxia led to a marked lactacidosis in the brain and CSF and to a significant breakdown of phosphocreatine and ATP In both situations there was a rapid rephosphorylation of ATP and a rapid reoxidation of the cytoplasmatic $NADH/NAD^+$ ratio, a slow rephosphorylation of phosphocreatine and a very slow removal of the lactate accumulated in the intra and extracellular spaces However, after the arterial h

tension there were more longlasting changes in the phosphocreatine, lactate and bicarbonate concentrations in spite of initial changes which were much less marked than during the 3 min asphyxia period studied in the preceding paper These differences may be due to the fact that the tissue blood flow is decreased during hypotension but not during asphyxia, but the duration of the hypoxic stress must also be taken into account However, before any definite conclusions can be drawn regarding the inherent nature of the hypoxic stress it should be borne in mind that the present metabolic changes may mainly reflect metabolic alterations in boundary zones with a vulnerable blood supply, which are exposed to a disturbance of the oxidative metabolism much more profund than indicated by the analyses on the tissue as a whole

Both the present and the previous communication have shown that the cytoplasmatic NADH/NAD ratio normalizes very quickly after that a normal blood pressure, or a normal arterial oxygen tension, is obtained These results emphasize the necessity to analyse the intracellular pH_i' when cellular redox states are studied by means of lactate/pyruvate analyses (Granholm and Siesjo 1969) Thus in the present study a marked increase in the intracellular lactate/pyruvate ratio in the 2 and 5 min restitution groups was found in spite of normal $NADH/NAD^+$ ratios There was also an apparent discrepancy between the CSF lactate/pyruvate rate and the cytoplasmatic NADH/NAD ratio It is thus clear that if the CSF lactate/pyruvate ratio shall be used for the evaluation of intracellular redox changes (see Siesjo, Kjallquist and Zwetnow 1968) the pH_i' changes must be calculated The finding of a parallellism between the increase in the CSF and intracellular lactate concentrations (see 10 and 20 min restitution groups in Fig 3), and the close relation between the increase in the intracellular lactate concentrations, and the decrease in pH_i' (see below), suggests that such calculations may eventually be feasible

It is clear from the present results, and from those of the previous communication (Kaasik *et al* 1969) that it is adventurous to equate the normalization of the tissue metabolism after a hypoxic episode with the normalization of the NADH/NAD ratios, or of the ATP concentrations Thus, neither the phosphocreatine concentrations nor the lactate and pyruvate concentrations or the pH values were normalized until much later and it is indicated that the sum of the adenine nucleotides and the ADP concentrations are not normalized *pari passu* with the ATP concentrations

There are reasons to believe that in a hypoxic or posthypoxic situation with moderate changes in the tissue concentrations of phosphocreatine and adenine nucleotides, the lactic acid accumulated is the main cause of the pH_i' changes observed This would mean that the relation shown in Fig 5 allows the calculation of the van Slyke buffer value (β) according to the equation

$$\beta = \frac{\Delta B}{\Delta pH} \quad (1)$$

where the Δ lactate is inserted as −ΔB A simple calculation from the line drawn in Fig 5 gives the value 0 042 moles per kg and pH unit The meaning of this figure will be evident if we calculate the corresponding buffer capacity of whole blood *in vitro* Blood has a high CO_2 buffer capacity due to the concentration of imidazole and α amino groups and Edsall and Wyman (1958) have shown that its buffer capacity to CO_2 can be mimicked by considering an imidazole buffer with a total concentration of 0 050 mole/l We can calculate the buffer value of an imidazole/bicarbonate buffer mixture from the equation

$$pCO_2 = \frac{(H^+)\left[BB - \frac{C\ K_{HA}}{(H^+)+K_{HA}}\right]}{S\ K_1'} \qquad (2)$$

(see Edsall and Wyman 1958, Siesjo and Ponten 1966 Messeter Ponten and Siesjo to be published) In this equation BB is the buffer base concentration C the total concentration of the imidazole buffer, K_{HA} the ionization constant of the buffer acid S the CO_2 solubility, and K_1' the ionization constant of the carbonic acid (CO_2 + H_2CO_3) If we assume an initial pH of 7 09 (present control group) a total buffer concentration for the imidazole buffer (C) of 0 050 mole/kg and a pK_{HA} for the imidazole buffer acid of 7 40, the buffer base concentration at a pCO_2 of 40 can be calculated to 28 2 meq/l We can now calculate the new buffer base concentration for *e g* a 0 1 pH change in the acid direction and from the new buffer base concentration calculate the Δ buffer base This calculation shows that the imidazole buffer system yields a buffer capacity of 0 05 In other words the intracellular space of the brain appears to have a lower buffer capacity to nonvolatile strong acids at constant CO_2 tension than has a system equivalent to the blood imidazole buffer system This finding is in agreement with the assumption that the *in vivo* CO_2 buffer capacity of brain tissue which greatly exceeds that of the blood *in vitro* (Kjallquist, Nardini and Siesjo 1969 Kjallquist Messeter and Siesjo 1969 Granholm and Ponten 1969) is due to other factors than physicochemical buffering

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Potentiation of Adrenergic Cardiovascular Effects of Two Quaternary Nicotine Analogues by Reserpine

By

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Abstract

Euler, U S v and N-A Persson *Potentiation of adrenergic cardiovascular effects of two quaternary nicotine analogues by reserpine* Acta physiol scand 1970 78 459—464

The quaternary nicotine analogues *iso*-monomethylnicotinium bromide (IMN) and 1 methyl 3 [illegible] MPP) exert only weak effects on the blood pressure [illegible] ized and adrenalectomized cat in doses of 0 5—1 [illegible] reserpine i v the effect is strongly potentiated but [illegible] ed by desipramine whereas the pressor effect of nicotine is annulled by hexamethonium, the action of the two quaternary compounds is markedly potentiated The effects of IMN and MPP are also enhanced by previous infusion of prenylamine 0 25 mg/min/kg It is concluded that reserpine allows IMN and MPP to get access to noradrenaline stores through the axonal membrane and cause a release of neurotransmitter

In a study of the pharmacological activity of a number of nicotine analogues (Haglid 1967, Euler *et al* 1969) it was noted that two of these, *iso* monomethylnicotinium bromide (IMN) and 1-methyl 3-(pyrrolidinomethyl) pyridinium bromide (MPP) in doses from 10 mg/kg upwards lowered the noradrenaline (NA) content of the heart and kidney of the guinea pig after intraperitoneal administration This effect was ascribed in part to a release of adrenergic transmitter caused by a nicotinic" excitation of adrenergic neurons presumably at the axon membrane and extragranular level since no effect was observed on the release of NA from isolated nerve granules (Euler *et al* 1969, Hedqvist 1969) The releasing effect was found to be quite slow, thus the maximal NA depletion after 10—30 mg/kg of IMN occurred 6 hrs after injection of the drug, higher doses requiring 12 hrs for the maximal effect to develop

Since previous studies have shown (Larson and Haag 1943, Barlow and Dobson 1955, Gillis and Lewis 1956) that IMN (in the form of the iodide) has only a weak action on the blood pressure of the spinal cat in mg doses it appeared of interest to study the acute effect of the two compounds on the cardiovascular system under dif-

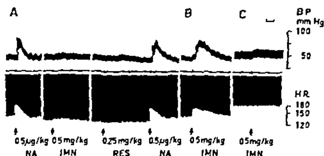


Fig 1 Cat spinal adrenalectomized, vagi cut. Upper curve blood pressure, lower curve heart rate I v injections as indicated Between B and C 2 mg/kg desipramine Time mark 1 min.

ferent conditions, involving interaction with certain drugs known to affect adrenergic neurotransmission (reserpine, hexamethonium, prenylamine and desmethylimipramine)

Methods

Cats were
Blood pre
Injections
respiration
ventilation

IMN and MPP *were administered as hydrobromides, reserpine* as Serpasil® (CIBA) desmethylimipramine (DMI) as hydrochloride and prenylamine as Segontin® (Höchst) and hexamethonium as bromide

We are indebted to Dr Frank Haglid for supplying IMN and MPP, to CIBA Products Vallingby and to Hochst Co Stockholm for generous gifts of Serpasil® and Segontin® respectively

Results

Cardiovascular effects of IMN and MPP on the spinal cat

On i v injection in the spinal cat the actions of IMN and MPP on the blood pressure and on the heart rate were generally small in doses of 0 5—2 mg/kg in conformity with earlier results (Larson and Haag 1943, Barlow and Dobson 1955, Gillis and Lewis 1956) (Fig 1—4) No difference in action was observed between the two compounds either qualitatively or quantitatively *In some animals showing a* high sensitivity to NA the two quaternary compounds caused a more marked rise in blood pressure after these doses

Reserpine

Reserpine in a dose of 0 25 mg/kg i v which had virtually no effect by itself on the blood pressure or heart rate caused a strong increase in the response to IMN as seen in Fig 1B, 2A 3D and 4C

Abbreviations IMN = iso monomethylnicotinium bromide MPP = 1 methyl 3 (pyrrolidinomethyl) pyridinium bromide hydrobromide NA = noradrenaline, TA = tyramine, DMI = desmethylimipramine, C_6 = hexamethonium bromide

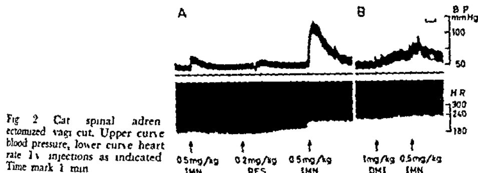


Fig 2 Cat spinal adrenectomized vagi cut. Upper curve blood pressure, lower curve heart rate I v injections as indicated Time mark 1 min

The weak action of IMN on the heart rate was also markedly enhanced after reserpine The same kind of potentiating effect of reserpine was observed for MPP

Hexamethonium

Hexamethonium 5 mg/kg, which abolished the pressor effect of nicotine on the blood pressure, enhanced the small effects of IMN and MPP on the blood pressure and heart rate (Fig 3C) The response was further increased by a following dose of reserpine 0 25 mg/kg i v (Fig 3D)

Prenylamine

Infusion of prenylamine 0 25 mg/kg/min regularly caused a slight increase in blood pressure (*cf* Euler and Lishajko 1968) After the infusion the effect of IMN and MPP were markedly enhanced (Fig 4B) A comparable increase in the response to NA 0 25 μg/kg/min and to TA 20 mg/kg was also noted after prenylamine, suggesting that the effect was due to an increased action of the NA released When reserpine was given 1/2 hr after prenylamine it caused a sustained rise in blood pressure A following dose of IMN now produced a still larger rise in blood pressure than seen before reserpine (Fig 4C) while the effect of NA was unchanged indicating that reserpine had permitted the release of a larger quantity of active NA by IMN

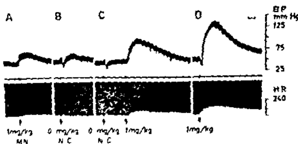


Fig 3 Cat spinal adrenectomized vagi cut Upper curve blood pressure lower curve heart rate I v injections as indicated Between B and C mg/kg hexamethonium bromide between C and D 0 mg/kg Serpasil® Time mark 1 min

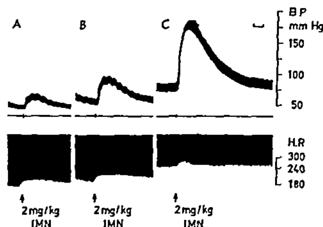


Fig 4 Cat spinal adrenalectomized vagi cut. Upper curve blood pressure, lower curve heart rate I v injections as indicated. Between A and B 0 25 mg kg Segontin®, between B and C 0 25 mg/kg Serpasil® Time mark 1 min

Desipramine

In order to obtain further information on the cause of the catecholamine releasing action of the two quaternary compounds we have studied the effect of desipramine (DMI) on the action of IMN and MPP after reserpine, since DMI inhibits the uptake of various amines at the axon membrane as well as at the granular level (Cuenca, Salva and Valdecasas 1964, Euler and Lishajko unpubl observations) It was found that after DMI in a dose of 0 5—2 mg/kg i v , which prevented the blood pressure rise after tyramine, IMN and MPP had only a small or no action in the same doses as were strongly active before desipramine (Fig 1C and 2B) This result suggests that DMI blocks the access of IMN and MPP to the NA stores, preventing the interaction normally leading to release of NA after reserpine

Discussion

From the results recorded in the present paper it appears that the weak cardiovascular effects exerted by the two nicotine analogues examined, under certain conditions can be strongly potentiated

The 'unmasking of the effect of the quaternary compounds on the cat's blood pressure and heart rate by reserpine suggests that the two compounds cause a release of noradrenaline from a site where it is largely protected under normal conditions but made accessible by reserpine This site is not represented by the storage granules, since no releasing action is seen on isolated nerve granules (Hedqvist 1969) It appears therefore that the compounds studied release the amine from an extra granular site possibly involving the axon membrane itself This effect is prevented by desipramine which blocks the entrance of various compounds through the axon membrane

The mechanism of the permissive action of reserpine is still unclear The results indicate, however, that it differs from that of prenylamine in that the latter drug

enhanced the action of injected NA as well as that of the quaternary compounds Since reserpine and prenylamine appear to act similarly at the granual level, the difference in action must be located either to the extragranular, intraaxonal storage system or to the axon membrane itself It has been reported that reserpine affects the membrane system of mitochondria (Wilcken *et al* 1967), and it appears conceivable that an action of this kind may make the extragranular transmitter storage system more susceptible to the releasing action of the compounds studied

Apparently reserpine itself does not release active transmitter from the axonal membrane since it exerts no sympathomimetic action without the aid of other drugs such as MAO inhibitors (Chessin *et al* 1957) The MAO inhibitors are believed to preserve the NA 'released' by reserpine Since no such precaution is required for the permissive action of reserpine in combination with the quaternary compounds studied it appears that these release the transmitter from a peripheral store where it is not inactivated by MAO The supposition that they may have a MAO inhibitor action receives no support from the observations of Yamamoto Nagai and Inoki (1966) who found no such action with nicotine Reserpine may therefore alter some structures at or close to the axon membrane allowing the transmitter to be more readily released by the quaternary compounds studied, without interaction of MAO

It should be recalled in this context that Zaimis (1961) has pointed out some effects of reserpine which support the notion that this drug may affect intracellular structures as indicated also by the observations on mitochondria At any rate it is hard to avoid the conclusion that the transmitter storage system has been profoundly altered by reserpine in the doses employed

Normally the transmitter escaping after reserpine is effectively inactivated by MAO (Kopin and Gordon 1962) and causes no effect while the quaternary nicotine analogues studied allow an increased release of active transmitter after reserpine, possibly from another location The results with desipramine suggest that the quaternary compounds and reserpine act in combination at a site associated with the axon membrane

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The Influence of Some Cholinergic Drugs and of Atropine on o-Iodohippurate Accumulation in Rat Renal Cortex Slices

By

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Abstract

BÁRÁNY, E. H. *The influence of some cholinergic drugs and of atropine on o-iodohippurate accumulation in rat renal cortex slices.* Acta physiol. scand. 1970. 78. 465—470.

The effect of some drugs on the accumulation of o-iodohippurate ^{125}I at 1.2 · 10^{-5} M in slices of rat kidney cortex [illegible] ... is non-cholinergic.

A transport system for organic acids in the ciliary processes of rabbit eyes was discovered by Becker (1960). The system is closely related to the hippurate excretory system of the kidney but for the fact that in the eye the transport is towards the blood. Since in the treatment of glaucoma autonomic drugs are applied to the eye in extremely high concentrations and might affect clinically important transport systems, Wålinder (1966, 1968) has studied the effects of several drugs used in glaucoma on different transport systems in the rabbit and monkey eye. One of his findings is that pilocarpine affects the accumulation of iodopyracet (which is carried by the hippurate system) by rabbit ciliary processes in vitro. Concentrations 10^{-7} to 10^{-5} M somewhat increased accumulation while 10^{-3} and 10^{-2} M were strongly inhibitory. Even this last concentration can be reached (at least in the anterior chamber) under clinical conditions and is therefore not quite unrealistic. Wålinder's experiments were not designed to test if the effects observed are related to the cholinergic action proper of the drug.

Because of the similarity of the hippurate transport system in the eye and the kidney, it seemed worthwhile to check whether pilocarpine also inhibits hippurate accumulation in slices of rat renal cortex using the Cross Taggart (1950) technique.

This was found to be the case Arecoline, eserine with acetylcholine and eserine alone were much less effective and the pilocarpine effect was not blockable by atropine It is concluded that the pilocarpine effect is not a true cholinergic one Direct cholinergic effects on this transport system of the kidney are slight if they exist at all.

Methods

Male Sprague Dawley rats, around 300 g from Anticimex Sollentuna Sweden were used. They were fed Anticimex rat pellets no 210 Under ether the left kidney was removed and very quickly sliced at about 0 5 mm in a Stadie Riggs slicer modified to allow quicker removal of the slices These were immediately placed in open Petri dishes containing a few mm depth of basal incubation fluid exposed to the air The dishes were supported in a water bath and their temperature was 37° C Cortex was dissected from medulla in these dishes After exactly 10 min in the dishes the cortex slices (each 15—20 mg wet weight) were transferred to pre-warmed 25 ml conical flasks containing 5 ml incubation medium and drugs The flasks had been gassed with O_2 and stoppered They were gently shaken at 38° C for 30—36 min Twelve cortex slices were used and each flask contained two or three slices Each run comprised 1 or 2 control flasks and 4—5 drug flasks The schedule was arranged symmetrically in time No other attempt was made to compensate for small differences in incubation time

After the incubation the slices were fished out of the flasks individually rapidly blotted, put into small preweighed boats of 0 01 mm aluminium foil, weighed on a torsion balance to the nearest 0 01 mg and dried over night at 45° C The slices were transferred in their boats to gamma counting tubes and counted in a Packard gammaspectrometer Measured volumes of medium were transferred to small pieces of cellulose sponge weighing about the same as dry cortex samples put into boats dried and counted exactly as tissue pieces

Bottles without radioactivity but with all drugs added were always run to check the pH which was around 7 4 Preliminary experiments had shown that slices recovered and kept better in Reynard s K rich saline than in the more conventional one The basal incubation medium therefore was K-rich and phosphate buffered to pH 7 4 Each liter contained besides 1 g glucose the following salts in millimoles 102 NaCl 37 KCl 1 16 Mg SO_4, 2 50 $CaCl_2$ 2 4 Na_2HPO_4 0 58 KH_2PO_4 All were of analytical quality In some experiments of Table I the amount of KCl was reduced to 1/8 and a corresponding amount of NaCl added This made it into Robinson s kidney solution All solutions were adjusted to pH 7 4 with a little HCl or NaOH if necessary

Sodium o-iodohippurate labelled with ^{125}I was obtained from the Radiochemical Centre Amersham It will be called Hippuran for shortness in the following The original solution contained 15 mg/ml Hippuran and 10 mg/ml benzylalcohol It was diluted with saline so as to contain 0 44 mg/ml Hippuran and 50 µl of this were added to 5 ml of incubation medium The strength in the incubation fluid was 1 2 10^{-6} M of Hippuran and 2 7 10^{-5} M of benzyl alcohol The chemical strength of the incubation fluid was kept equal throughout the experimental series Only in one group of experiments (Table II) was the solution 5 times stronger with respect to Hippuran and benzylalcohol

Alpha naphthuric acid has been prepared by Miss G Stallberg It was one of the most effective inhibitors of hippurate uptake in a long series of hippurate analogues (Barány unpublished) Drugs used were added in 50 µl to 5 ml incubation fluid They were pilocarpine HCl eserine salicylate arecoline HBr (Merck) atropine sulphate acetylcholine chloride (Roche)

In the calculations one incubation flask was the
one slice The medium in each flask was
mean and standard error of the per cent
from the ratio of each flask to its approp

Results

No attempt was made to estimate extracellular space in the slices Water content was on an average 77 2 % None of the drugs did affect it measurably Tissue/medium ratios of 5—7 on a wet weight basis were usually attained after 30 min in control slices

TABLE I Influence of pilocarpine on accumulation ratio AR of Hippuran in rat kidney cortex slices The row "%" shows AR with pilocarpine in % of simultaneously run controls Number of flasks with each concentration (= n) is statistical unit There were 2—3 cortex slices in each flask Their AR was averaged Hippuran concentration 1 2 × 10 [illegible] M Means ± s e m

Medium		Pilocarpine molarity				
		0	10 [illegible]	10 [illegible]	10 [illegible]	10 [illegible]
Standard K rich	AR	5 38 ± 0 20	5 33 ± 0 17	4 91 ± 0 27	4 24 ± 0 32	3 31 ± 0 25
	n	12	6	6	6	6
	%	100	103 1 ± 3 7	95 7 ± 7 5	78 8 ± 5 2	60 9 ± 6 2
Robinson s kidney solution	AR	2 63 ± 0 14				1 52 ± 0 12
	n	12				12
	%	100				58 1 ± 3 9

Part of the Hippuran-uptake in a slice is not due to the anion transporting system A commonly used way to estimate this part is to run slices at 0° C or during anoxia Since these procedures change the proportion of extracellular space they are not ideal In the present experiment saturation of the specific transport system with alpha naphthuric acid was used instead In the presence of 8 8 10 [illegible] M of this anion slice/medium ratios for Hippuran were 1 00 at 30 as well as at 60 min Accordingly accumulation ratios AR as shown in the tables were taken to be slice/medium ratio minus 1 0 Percent uptake in drug experiments is also calculated using slice/medium ratios minus 1 0 This figure was obtained from experiments with 1 2 10 [illegible] M Hippuran and might be slightly different with a different concentration

TABLE II Influence of pilocarpine on accumulation ratio AR of Hippuran at two Hippuran concentrations Further explanation see Table I

Pilocarpine molarity	0		10 [illegible]	
Hippuran molarity	1 2 × 10 [illegible]	6 × 10 [illegible]	1 2 × 10 [illegible]	6 × 10 [illegible]
AR	4 34 ± 0 36	2 30 ± 0 21	2 60 ± 0 13	1 51 ± 0 09
n	10	10	10	10
% of control at 1 2 × 10^{-5}	100	53 0 ± 2 5	61 7 ± 2 9	
% of control at 6 × 10 [illegible]		100		68 1 ± 3 6
			Difference 6 4 ± 3 9 Not significant Ratio 1 120 ± 0 065 Not significant	

TABLE III Influence of atropine and pilocarpine on accumulation ratio AR of Hippuran. For further explanation see Table I

Pilocarpine molarity		Atropine molarity				
		0	3 5 × 10 [illegible]	3 5 × 10 [illegible]	3 5 × 10 [illegible]	3 5 × 10 [illegible]
0	AR	5 35 ± 0 12	4 63, 5 53	5 64, 5 85	5 75, 5 77	
	n	6	2	2	2	
	%	100	90	108	114	
0	AR	5 28 ± 0 44			5 59 ± 0 54	
	n	5			5	
	%	100			105 6 ± 3 4	
10^{-3}	AR	3 32 ± 0 29			3 74 ± 0 31	
	n	5			5	
	%	62 8 ± 1 3			69 9 ± 5 4	
0	AR	4 91, 4 63				4 92, 4 58
	n	2				2
	%	100				100
3 3 × 10 [illegible]	AR					2 83 ± 0 27
	n					4
	%					59 5 ± 4 2

Table I shows that pilocarpine reduces the uptake of Hippuran in a dose-dependent manner A significant reduction begins above 10^{-5} M With 10^{-3} M the percentage uptake is very much the same in the standard K-rich medium (60 9 ± 6 2) and in one that contains a more physiological amount of potassium (58 1 ± 3 9) At a fixed concentration of pilocarpine the inhibition is similar at two concentrations of Hippuran which differ 5 fold (Table II) Special experiments with benzylalcohol showed that the lower accumulation ratio at the higher Hippuran concentration (2 30 as against 4 34) was not due to the benzylalcohol The % AR with benzyl alcohol at the higher concentration compared with the lower one was 86 0 ± 8 0 (n = 12) Thus the half saturation point for Hippuran in our system is around 6 10^{-5} M

Table III shows that atropine even in high concentrations had little effect on accumulation ratios and did not prevent the effect of pilocarpine This is especially evident in the last row of Table III where the molar concentration of atropine is 10 times that of pilocarpine

The failure of atropine to inhibit the pilocarpine effect suggests that either pilocarpine does not act as a cholinergic drug in this system or that atropine has a very low affinity to the relevant receptors The effect of acetylcholine in the presence of eserine was therefore tested Table IV shows that eserine salicylate has a weak effect

TABLE IV Influence of acetylcholine and eserine salicylate on accumulation ratio AR of Hippuran Symbols and conditions as in Table I

Molarity of eserine salicylate		Molarity of acetylcholine						
		0	10 [illegible]	10 [illegible]	10 [illegible]	10 [illegible]	10 [illegible]	10 [illegible]
0	AR	5 52 ± 0 17						
	n	14						
	%	100						
24 × 10 [illegible]	AR	4 92 ± 0 27						
	n	7						
	%	90 5 ± 3 3						
24×10^{-4}	AR	4 74 ± 0.20						
	n	7						
	%	84 9 ± 3 3						
24 × 10 [illegible]	AR	4.26±0 11			4 10±0 08	3 84±0 14	3 95±0 14	3 82±0 12
	n	8			4	4	4	4
	%	100			92 2±4 3	86 2±2 7	97 7±4 4	94 3±3 5
24 × 10 [illegible]	AR	4 26±0 16	3 96 4 10	4 44 4 29	4 45 4 82	4 03 4 03		
	n	4	2	2	2	2		
	%	100	101	110	102	89		

TABLE V Influence of arecoline and atropine on accumulation ratio AR of Hippuran For further explanation see Table I

Atropine molarity		Arecoline molarity				
		0	10 [illegible]	10 [illegible]	10 [illegible]	10 [illegible]
0	AR	5 09±0 24	5 27±0 38	5 68±0 76	4 80±0 43	4 ?7±0 44
	n	12	6	6	6	6
	%	100	101 3±8 2	109 3±6 3	98 0±7 6	86 5±4 7
35 × 10 [illegible]	AR	5 60 4 23				3 51±0 38
	n	2				4
	%	100				71 1 3 9

in itself possibly partly due to the salicylate anion but that acetylcholine over a wide range has very little or no effect

Finally another tertiary cholinergic was tested namely arecoline Table V shows that there is a slight effect at 10^{-3} M and that this is not abolished by 3 5 10^{-3} M atropine The arecoline effect is markedly smaller than that of pilocarpine despite

the fact that arecoline is a considerably more active cholinergic than pilocarpine (van Rossum 1962)

It seems fair to conclude that the pilocarpine effect is not cholinergic

Discussion

The starting point of this investigation was ophthalmological Since no experiments on ciliary processes have been made in the present series the possibility remains that in the eye the inhibitory effect of pilocarpine on the hippurate system is at least *partly cholinergic But this does not seem probable The inhibition found by* Wålinder with 10^{-3} M pilocarpine was about 50 %, in the present experiments it was about 40 % The difference is not large and leaves little room for a specific effect The stimulating effect found by Wålinder (1966) at 10^{-6} M—10^{-5} M was not seen here

From the point of view of renal pharmacology the results presented may have some relevance in connection with the search for cholinergic control of tubular secretion (see *e g* Parmelee and Carter 1968) First there is no evidence of cholinergic receptors in the hippurate transport system This agrees with the fact that no cholinergic innervation of the proximal tubules has been reported (Doležel 1958) But the results also show that the use of PAH extraction as a measure of renal plasma flow in experiments dealing with high concentrations of pilocarpine or arecoline might give misleading results

The mechanism of the pilocarpine effect is not clear Opening of the lactone ring of the drug would convert pilocarpine into an acid and conceivably this acid might compete for transport with Hippuran The experiments with higher doses of Hippuran (Table II) were made to check this point If pilocarpine or a pilocarpine metabolite were a competitor in the transport system the effect would have been much smaller at the higher Hippuran molarity This was not the case

This investigation was supported by Grant K68 14X 733 03B from the Swedish Medical Research Council The excellent technical assistance of Mrs Malin Svensson is gratefully acknowledged

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From the Department of Biophysics, University of Copenhagen Denmark

Intracellular Potentials of the Corneal Epithelium

By

NIELS EHLERS

Received 13 June 1969

Abstract

EHLERS, N *Intracellular potentials of the corneal epithelium* Acta physiol scand 1970 78 471—477

The potential and [illegible] by mea[illegible] A trans[illegible] 15 mV [illegible] epithelium, which reached but never exceeded the level of the inside potential The number of these peaks increased when epithelial oedema was produced by increasing the transcorneal hydrostatic pressure difference In consequence the peaks are believed to represent intercellular spaces The electrode resistance was measured continuously and simultaneous with the potential. During penetration of the cytoplasm the resistance was about twice as high as in the bathing fluid, it increased further when cellular membranes were punctured, and especially when the basement membrane was penetrated

An electrical potential difference of 15—20 mV exists across the rabbit cornea with the epithelial side negative in relation to the endothelial (Donn Maurice and Mills 1959, Modrell and Potts 1959, Friedman and Kupfer 1960 Itoi, Komatsu and Tanda 1964, Ehlers and Ehlers 1966, 1968 Maurice 1967) This potential difference is maintained by the epithelium, and abrasion causes an immediate fall in the potential The orientation of the potential and the direction of the generating current have been considered important in the problem of maintenance of normal corneal turgescence and transparency

Kikkawa (1964) and Okuhara and Kikkawa (1967) are the only investigators who have studied the intraepithelial potentials by means of microelectrodes, and in rabbits found negative intracellular potentials increasing in magnitude from the surface to the basement membrane where a jump in positive direction was found However, the potential was still negative in relation to the surface Considering this result, which indicates a direction of the transepithelial potential opposite to that found in all above-mentioned studies it was found to be of interest to reinvestigate the profile of the potential and the electrode resistance while penetrating the epithelium and superficial stroma with a microelectrode

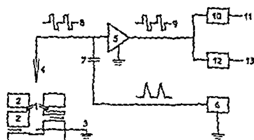


Fig 1 The cornea (1) is mounted between the perspex chambers (2) The temperature regulation by circulating water is not shown. The lower chamber is grounded via an Ag AgCl electrode (3) The micropipette (4) is moved by a micromanipulator (not shown) and the movement recorded by a potentiometer coupled to the micrometer screw The Ag AgCl electrode of the micropipette is connected to the input of a negative capacitance electrometer (5). Triangular voltage pulses from a function generator (6) are led to the input via a capacitor (7) resulting in diphasic currents (8) flowing through the micropipette to ground, and a diphasic output (9) from the preamplifier proportional to the electrode re... The output is a) passed through a f... the output to be recorded (11) ... value of the AC component of ... electrical potential and the resi... pipette

Methods

Corneas from rabbits (8) and cats (7) were isolated as previously described (Ehlers and Ehlers 1966, 1968) and mounted between perspex chambers (Fig 1) The lower endothelial side chamber was closed and the hydrostatic pressure, corresponding to the intraocular pressure, was controlled by the height of a fluid reservoir The fluid in this chamber was continuously renewed The upper chamber was open and directly accessible for the microelectrode The ... was surrounded ... 32° C. In all ... 46 2 meq Na⁺, ... meq H_2PO_4, ... cent CO_2 ... dified ... 120– ... Knudsen 1968), and the impedance was about 10 M Ω measured in the bathing fluid before and after penetration of the tissue

The measuring circuit is shown in diagrammatic form in Fig 1 The endothelial side chamber was grounded via an Ag—AgCl electrode The Ag—AgCl electrode of the micropipette was connected to a negative capacitance electrometer input stage (Keithley type 605) Intermittent triangular voltage pulses were differentiated by a small capacitor connected to the input (Lettvin, Howland and Gesteland 1958 The diphasic rectangular current pulses flowing ... voltage proportional to the joint resistance to the micropipette The preamplifier output con... ...uare waves with amplitude proportional to the ... a filter removing the square pulses and per... ...ectified by a Hewlett Packard 410 C voltmeter measuring the numerical value of the AC component of the signal giving as output a DC signal proportional to the electrode resistance ... ...ement was recorded ... ne for a ... n of the ... a dual

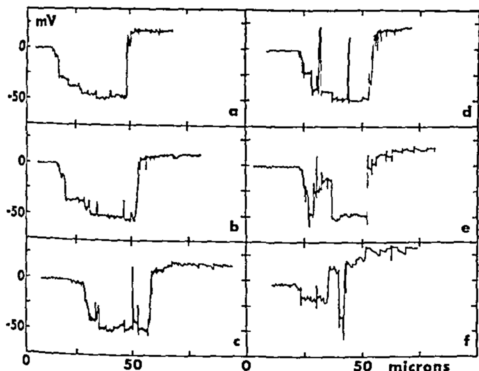


Fig 2 Typical potential recordings from penetrations of rabbit corneas Abscissa position of the micropipette in μ Ordinate potential in mV The time of penetration is about 30 secs The electrode is moved from left to right In a b and c the transcorneal hydrostatic pressure was 18 mm Hg in d 29 mm Hg and in e and f 37 mm Hg In experiments e and f the endothelium had been scraped off before the cornea was mounted in the perspex chamber

Results

1 Experiments on rabbit corneas

The changes in potential in the majority of penetrations were recorded as shown by the examples in Fig 2 a, b A steady potential was recorded in the bathing fluid and when the surface was penetrated a fall of 46 65 ± 1 07 mV (mean ± S E M 32 penetrations) was seen This fall often occurred over a distance of 5—10 μ after which no regular changes were seen until at a depth of 39 0 ± 0 9 μ a sudden change of 58 25 ± 1 19 mV in positive direction was found thus giving an overall trans epithelial potential of about 12 mV with inside positive No potential changes were recorded when the outer 100 μ of the stroma was penetrated The profile did not differ when central or peripheral cornea was penetrated neither was it immediately affected by changes in the transcorneal hydrostatic pressure within the range 10—80 mm Hg

In some penetrations the initial fall in potential occurred in two or more steps (Fig 2 b c) A gradual fall in potential when the electrode penetrated the epithelium was seen only with thick low resistance electrodes

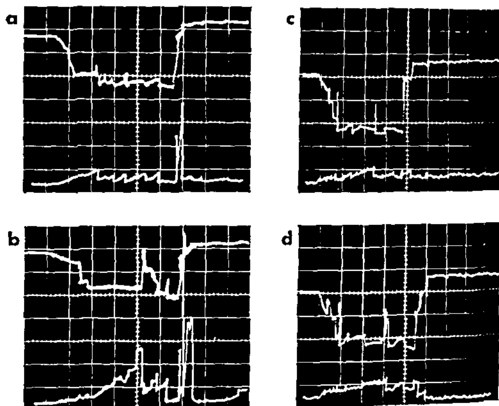


Fig 3 Photographic recordings of potential and electrode resistance during penetration of rabbit corneas Abscissa position of micropipette, 8 3 μ/div Ordinate Upper trace potential 20 mV/div lower trace electrode resistance 10 M Ω/div In a and b the transcorneal hydrostatic pressure was 18 mm Hg in c and d 70 mm Hg

In 45 per cent of the penetrations positively directed peaks were also recorded within the epithelium (Fig 2 c d) The peaks could reach the potential level of the stromal side but this was never exceeded The width and the number of the peaks increased when the transcorneal hydrostatic pressure had been elevated for 1/2—1 hr from the normal 15—20 mm Hg to 40—80 mm Hg and particularly when the endothelium had been scraped off (Fig 2 e, f)

The resistance of the electrode was approximately doubled when the tip touched and penetrated the epithelium Within the epithelium small peaks in positive direction were often seen (Fig 3 a) probably indicating puncture or touching of cellular structures A fall in resistance was the typical finding when a potential peak was recorded (Fig 3 b) Corresponding to the penetration of the basement membrane an increase in resistance to 40—100 M Ω was generally found (Fig 3 a b) On further penetration the resistance fell, and remained low except for artificial increase due to blocking of the electrode tip

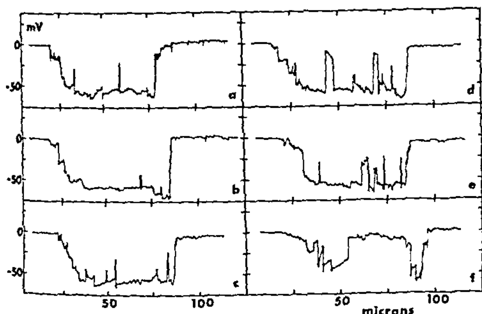


Fig 4 A sequence
Ordinate potential
static pressure 20 n
The pressure had
intervals

2 Experiments on cat corneas

The profiles recorded were fundamentally similar to those of the rabbit (Fig 4 a—f) The potential primarily showed a fall of 41.56 ± 0.68 mV (mean ± S E M, 41 penetrations) and at a depth of 56.7 ± 1.5 μ it increased by 43.24 ± 0.68 mV thus giving no statistically significant transepithelial potential difference Through the outer 100 μ of the stroma no potential changes were recorded The profile did not differ when central or peripheral cornea was penetrated neither was it affected by changes in the transcorneal hydrostatic pressure As in the rabbit experiments the initial fall in potential sometimes occurred over a certain distance (Fig 4 c d) and sometimes in two or more steps (Fig 4 a b) An oblique profile through the entire epithelium was seen only with thick low resistance electrodes

Peaks similar to those seen in the rabbit were recorded in 54 per cent of the penetrations Their width and number increased when the transcorneal hydrostatic pressure was elevated (Fig 4 a—f)

Also the changes in electrode resistance were similar to those seen in the rabbit The resistance was approximately doubled when the electrode touched and penetrated the epithelium Small peaks in positive direction were seen in the epithelium probably indicating puncture or touching of cellular structures When potentials peaks were recorded the resistance was as a rule lower than in the surrounding tissue An

increase in electrode resistance to 40—100 M Ω was generally seen, corresponding to the penetration of the basement membrane Further penetration did not show changes in resistance

Discussion

A regular potential profile has constantly been recorded when the corneas of rabbits and cats were penetrated from the surface with a microelectrode An initial fall in potential was found of 47 mV in the rabbit and 42 mV in the cat As a rule the total negative value was recorded within 5—10 μ of the surface The potential then remained fairly constant until an increase of 58 mV in the rabbit and 43 mV in the cat was observed corresponding to the base of the epithelium Further penetration showed no potential changes

The epithelial layer is very regular, having a thickness of 30—40 μ in the rabbit and 35—50 μ in the cat (Ehlers 1969) From this anatomical regularity there is no doubt that the second change occurs at the base of the epithelium In some penetrations the initial fall occurred in two or more steps, but in nearly all penetrations a plateau was reached Oblique potential profiles through the entire epithelium were seen only with thick low resistance electrodes The stepwise fall in the superficial part of the epithelium may be explained by reduced membrane potentials of the cells before desquamation In some few penetrations the potential change at the base of the epithelium was recorded in two or more jumps (Fig 2 b d 4 a f) This might indicate that one potential jump occurs across the plasma membrane of the basal cell and another presumably a diffusion potential, across the extracellular basement membrane It may however also be due to electrode tip potentials during penetration of the basement membrane

A transepithelial potential of 10—15 mV was found in the rabbit and in agreement with studies using macroelectrodes the inside was positive in relation to the outside In the studies of Kikkawa (1964) and Okuhara and Kikkawa (1967), only the outer 50 μ of the cornea was penetrated A simple explanation why the transepithelial potential was found to be reversed with the inside negative might be that the electrode did not penetrate the stroma at all

No statistically significant transepithelial potential difference was found in the cat. Seven human corneas were studied previously (Ehlers and Ehlers 1968), and no transepithelial potential could be demonstrated despite the use of various bathing fluids It is therefore still an open question whether a transepithelial potential occurs generally among mammals During the present experiments negative intracellular potentials have been verified in corneas from dog ox and rat The transepithelial potential appear as the difference between the two potential steps in the box , each of which is influenced by the ionic composition of the bathing fluids In accordance with studies on frog skin (Ussing and Windhager 1964) the transepithelial potential in rabbits is sensitive to the sodium concentration on the outside and the potassium concentration on the inside (Muneoka 1967 Ehlers unpublished observations)

When a potential peak was recorded a fall in resistance was the rule The peaks reached the level of the inside potential, but this was never exceeded The number and the width of the peaks increased when the transcorneal hydrostatic pressure was increased This procedure is known to dilate the intercellular spaces in the rabbit corneal epithelium (Ehlers and Ehlers 1968) For these reason it is probable that the peaks represent intercellular spaces in direct communication with the inside i e the stroma in accordance with the general concept of squamous epithelia (Ussing and Windhager 1964, Schoffeniels 1967)

The present experiments have been supported by grants from the Danish Foundation for the Advancement of Medical Science

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Long-Term Effects of High Intensity Flashes on the ERG of the Rabbit

By

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Abstract

KNAVE, B *Long-term effects of high intensity flashes on the ERG of the rabbit* Acta physiol scand 1970 78 478—490

A method was developed allowing long term studies on retinal function in the unanesthetized rabbit High intensity flashes were found to cause long-lasting reversible excitability changes in the retina Even a single flash with an intensity of about 7 log units above the electroretinographic *b*-wave threshold of [illegible] which lasted for about a week F [illegible] crease followed by a long-term de [illegible] corresponding reversible changes [illegible] The effects described are not due to influences on a shielding pigment or to a central efferent mechanism since the flash effects were also obtained on albino rabbits and after intracranial section of the optic nerve

In several investigations it has been shown that two kinds of visual adaptation occur, one neural, the other photochemical (Granit 1947, Rushton et al 1955, Elenius 1958, Rushton 1961a, b, Dowling 1963, Rushton 1965a, b) After bright light the dark adaptation consists of an initial rapid neural phase followed by a slower photochemical one After dim light only neural adaptation occurs The site of the neural adaptation has been referred to the cells of the inner nuclear layer of the retina (Brown and Watanabe 1965, Dowling 1967a) According to Dowling and Boycott (1966) the reciprocal synapses between the bipolar terminals and the amacrine processes could account for this adaptation by a mechanism of inhibitory feedback on the bipolar cells

Hagins (1955) found that a brief electronic flash, however bright, would not *bleach away more than about half the rhodopsin from a rabbit's retina* This finding was analyzed in experiments where the thermal decomposition of rhodopsin in solution was arrested by cold (Hubbard and Kropf 1958, Kropf and Hubbard 1958) It was found that a brief high intensity flash not only isomerized the 11-*cis* isomer of retinene but that it could also isomerize all-*trans* either back to 11-*cis* or to other isomers

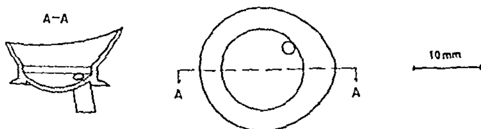


Fig 1 Contact lens for ERG recordings from the intact rabbit eye The active electrode inserted into the tip of the lens is placed limbally on the cornea when the contact lens is applied to the eye

Dowling and Hubbard (1963) studied the effect of flashes on dark adaptation in the rat They found that a short brilliant flash which photogenerates about 35 % photopigment raises the electroretinographical threshold to a level which corresponds roughly to the presence of this amount of visual pigment During the first 20—30 min following the flash, however, rhodopsin accumulates very slowly After about 30 min in the dark the photopigment regeneration and threshold curves join the recovery curves from a much higher state of prolonged light adaptation Similar observations have also been made in experiments on man (Rushton 1963)

A delay in photosensitivity after bright flashes has also been described in the human eye (Weale 1965) and in electroretinographic studies on humans on the effect of high intensity double flashes (Elenius 1967) In the rat a suppression of the ERG was noted over a 12 hr period following an electronic flash (Buckser 1966)

In this context it should be mentioned that more than 20 years ago it was found that prolonged exposure to sunlight in natural conditions produces temporary and cumulative effects on night vision (Hecht *et al* 1948) For example after several hours' adaptation to sunlight on the beach a whole night of dark adaptation was not sufficient to bring sensitivity back to its previous level Several days spent at lower illumination were required to achieve this result

With this long lasting effect in mind a question arises Do electronic flashes, in addition to the delayed rhodopsin regeneration described above also elicit long term changes in retinal function? In order to make such studies possible a method was developed which allows long term ERG studies on unanesthetized rabbits In the present paper this method will be described as well as the long lasting excitability changes which could be followed for several weeks

Material and Methods

Material Adult pigmented Chinchilla rabbits (in one experimental series albino rabbits), weighing between 2 5 and 3 5 kg were used Corneal diameters were checked to be less than 15 mm to exclude eyes with glaucoma (Kolker *et al* 1963)

Prep

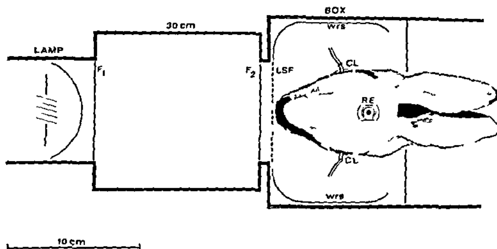


Fig 2 Schematic drawing of the experimental arrangement with flash lamp, connecting tube (30 cm) and rabbit box. *Abbreviations* CL *contact lenses F1 and F2 neutral filters.* LSF light scattering filter RE reference electrode wrs white reflecting screen

tropicamide (Mydriacyl®) eye drops Cornea and conjunctiva were anesthetized with 2 % tetracain eye drops The orbicular muscle was infiltrated with 2 % xylocain-exadrin (Astra) to prevent blinking The reference electrode was applied to mid line forehead skin The animal was then placed in a light tight and air ventilated box (see Fig 2) and the head was fixed in a head holder

Normally the rabbit can develop varying degrees of exophthalmos In the present material there was little or no exophthalmos In a few animals the exophthalmos required a slight anterior tarsorrhaphy in order to make the contact lenses fit the eyes

Recording system The two recording electrodes (one for each eye) and the reference ground electrode were all matched calomel half cells connected to the preparation by saline bridges in [illegible] area of 2.5 mm² and the reference [illegible] suitable pieces [illegible] The reference [illegible] of this tube [illegible]
The recording electrodes were inserted in the contact lenses and were always placed [illegible] and upwards on the cornea.

Both eyes were studied and each eye electrode was connected to a Grass P 6 DC preamplifier and to the amplifying systems of a Tektronix 502 dual beam cathode ray oscilloscope (CRO) In most experiments a capacitor was inserted between the preamplifier and the CRO to give time constants ranging from 10 to 1000 msec Throughout the experimental series an upper cut-off frequency of 2 kHz was used Pictures were taken with a Grass kymograph camera In some experiments a computer of average transients (Mnemotron Corporation type CAT 400 A) was connected to the output stage of the amplifier of the CRO The 400 analysis points of the computer were set to cover an analysis time of 125 msec The averaged signal was led to the CRO screen for photographing

The amplifying systems were regularly calibrated [illegible] were applied [illegible]

Light stimulation The test light was obtained from an electronic flash (Braun type F 82, Professional) According to the manufacturer this flash operates at 165 J and has a colour temperature of about 6500° K i e a spectrum similar to the solar spectrum. An investigation of the temporal distribution of the luminous flux by means of a phototransistor (Mullard type BP Y 25), showed a raise time of 100 μsec peak time of 140 μsec half time of 650 μsec and full time of 1200 μsec

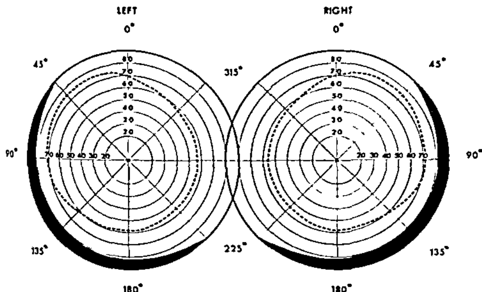


Fig 3 The rabbit vision fields as judged by ophthalmoscopic transillumination of intact eyes and after the application of contact lenses (broken line)

The flash was driven by a battery charged from the main Maximum discharge frequency was about 4 per min, at which frequency 20 consecutive flashes could be obtained In order to obtain 100 consecutive flashes a frequency of 2 per min had to be used

The lamp was placed 30 cm in front of the box (see Fig 2) Between the lamp and the box was

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0, an intensity attenuated to 1/10 as log relative intensity −1 (log10 0 1) etc The intensities of the test light were controlled by neutral density filters (AGRAFO)

In the present investigation the electroretinographic responses were measured from time to time in long term experiments and a comparison was made between the two eyes This means that certain facts concerning retinal illumination must be taken into account Thus, the retinal illumination must be the same and the two eyes must be equally illuminated Further a wide-angle stimulus field with uniform intensity is required in order to provide even retinal illumination It must be kept in mind that the retinal illumination does not only depend on the light stimulus but also on pupil size and opacities of the ocular media Changes in the pupil size were eliminated by the use of dilatating eye drops

On

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vision fields After application of a contact lens

ie stimulus field In Fig 3 the vision fields and

shown in the right and left eyes The relative

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located 5 cm below 5 cm behind and 3 5 cm inside the surrounding walls of the box In three series of investigations the phototransistors were placed in the horizontal plane and

other planes i e 45° upward and 45° downward from the horizontal plane Moreover they were placed in eight different outward directions in the horizontal plane, i e 35—40°, 60°, 80°, 100°, 120°, 140°, 160° and 180° from the sagittal plane, where 0° means straight forward and 180° straight backward This means that the illuminance of the light stimulus was measured at 144 points around the rabbits eyes It was shown that within the stimulus field the relative intensity did not vary more than ±0 1 log units Thus both eyes are equally illuminated by a wide angled, uniform light stimulus The constancy of the illumination of the repetitive flashes (maximal frequency of 2 flashes/min) was ascertained Further no change of the illumination was noted over a 3 month period

ERG records and determination of threshold responses A response of 20 μV was usually the smallest response that could be clearly separated from the noise The *b* wave potential chosen as 'threshold was 50 μV Amplitudes and latencies were taken as the mean of at least three consecutive determinations

Results

The electroretinogram elicited by low and high intensity flash stimuli

When using the flash light described above (see Method) the threshold of the *b*-wave was obtained when the flash was attenuated to 1 10^7 (log relative intensity −7)* The intensity had to be increased more than 2 log units before the *a* wave appeared Maximal *b* wave without any preceding *a* wave was obtained at log relative intensity −5

As shown in Fig 4 A the responses from the right (upper tracing) and the left eye (lower tracing) are similar in amplitude and shape The records in Fig 4 B show the responses from both eyes to a flash with an intensity 7 log units above the threshold of the *b* wave (log relative intensity 0) At this intensity the *a* wave (peak latency 4 msec) and subsequent *b* wave (peak latency 90 msec) are followed by a large remnant negativity (*cf* Granit 1959) and a long lasting *c* wave which are illustrated in Fig 4 C (slow sweep speed) The peak latencies of these deflections are about 0 5 and 3 sec respectively and the base line is not reached until about 10 sec after illumination

The records in Fig 4 A and B show small oscillations superimposed on the ascending part of the *b* wave the so called oscillatory potential (Cobb and Morton 1954 Heck and Rendahl 1957 Yonemura *et al* 1963) The amplitudes of the oscillations are known to increase under photopic conditions Fig 4 D—F shows the oscillations under photopic conditions (time constant 10 msec) Repetitive flashes with a frequency of two per minute provided a state of photopic adaptation The ERGs following 10 such repetitive flashes are superimposed in Fig 4 D The five positive oscillations seen in this figure are called o_1 o_2 o_3 o_4 and o_5 (o_1 is hardly visible as a positive oscillation between the *a* wave and the first negative wavelet) Two additional positive oscillations o_6 and o_7 were seen after increasing the signal to-noise ratio by computer averaging 100 recordings Fig 4 F shows an expanded amplitude display of the recording in Fig 4 E The peak latencies of the seven positive oscillations were 8, 14 20 30 39 52 and 65 msec respectively

Thus the results obtained with the computer technique are in accordance with the results of experiments with intraretinal microelectrodes and clamped retinal circula

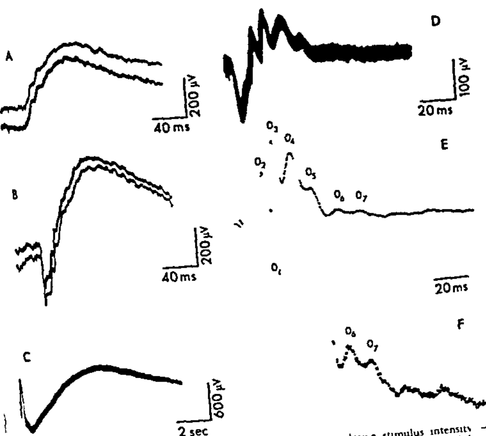


Fig 4A—C. The ERG of the dark adapted rabbit eye Log relative stimulus intensity −5 (A) and 0 (B and C) DC-amplification Records from right upper tracing and left eye (lower tracing) in A and B

Fig 4D—F The oscillatory potential of the rabbit ERG D 10 tracings superimposed E 100 recordings computer averaged F Expanded display of the curve in Fig 4 E Time constant 10 msec

tion (see Brown 1968 in which at least 10 oscillations successively fading away were recorded In these experiments made on monkeys it was shown that the oscillations passed through an amplitude maximum superficial to the inner nuclear layer and that they were critically dependent upon the retinal circulation These results substantiate the view that the oscillations are generated from reverberating circuits of the retinal neuronal network thus depending on a neural feedback mechanism As a matter of fact both physiological (Rushton 1965 a and morphological (Dowling and Boycott 1966) backgrounds for such a mechanism exist

Constancy of b wave of the electroretinogram elicited by a low intensity stimulus

In the investigation on the normal variations of the b wave with the technique described log relative intensity −5 was used (responses illustrated in Fig 4 A) Tab

shows the results obtained on three rabbits during a period of 6 weeks. The studies were made on dark adapted animals (see Method). Each value in Table I gives the average of the two values obtained by recording from the right and left eye (see Fig. 4 A). As can be seen, the standard deviations obtained in the experiments on the three animals were 5, 6 and 10 μV, respectively, corresponding to 1.8 and 2.9 per cent of the mean. The weighted mean of the standard deviations for a single observation was calculated, weighted $s_b = 7\ \mu V$, i.e. 2.3 per cent of the mean.

TABLE I. Normal variation of the *b*-wave amplitude

Rabbit No.	Amplitude (μV) at observation no.						$\bar{b}$ (μV)	s_b (μV)	s_b (%)
	1	2	3	4	5	6			
1	282	276	284	275	285	285	281	5	1.8
2	331	330	323	319	338		328	6	1.8
3	337	347	363	352	345		349	10	2.9

Here, reference should be made to the results reported by Spivey and Pearlman (1963). They studied the day-to-day variation of the *b*-wave in three rabbits and found a considerably greater variation than that of the present study. The difference is probably due to the differences in the methods used.

Long term changes in retinal function induced by high intensity flashes

As mentioned above, the illumination was about 100 Lux during preparation. After finishing the preparation, the illumination was turned off. The curve to the left in Fig. 5 shows the increase during darkness of the *b*-wave in response to a flash with the log relative intensity −5. As can be seen, the *b*-wave reaches maximal values after about 6 min. After 20 min a high intensity flash (log relative intensity

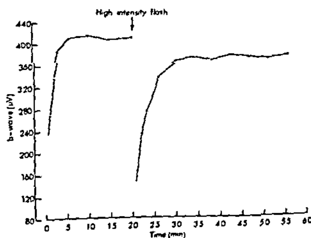


Fig. 5. The recovery in darkness of the *b*-wave of the rabbit ERG following a light adaptation of 100 Lux for 10 min (the curve to the left) and after an electronic high intensity flash given after 20 min in the dark (the curve to the right). The electronic flash had an intensity of about 7 log units above the *b*-wave threshold of the dark adapted eye.

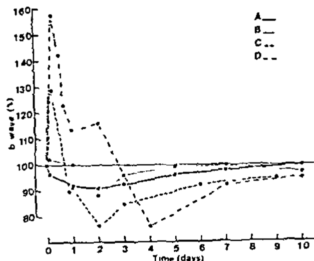


Fig 6 Amplitude of *b* wave (in per cent of pre illumination value) after illumination with single and repetitive (10 flashes in B 100 flashes in C and D) light flashes Intensity of illumination 7 (A and B), 8 (C) and 9 (D) log units above the *b*-wave threshold in the dark adapted eye

0) was given and the curve to the right shows that under these conditions the increase of the *b* wave is slower and that the maximal *b*-wave amplitude was not reached even after 40 min

The question then arises for how long a time may such a flash effect persist? In order to study this problem experiments were performed on four rabbits Curve A in Fig 6 shows the *b* wave amplitude changes after a single conditioning high intensity light flash and B, C and D the changes following a series of repetitive conditioning flashes (conditioning stimulus presented at zero on time axis) Also in these experiments the test flash had the log relative intensity -5 Each curve in the diagram represents a series of measurements on both eyes of one animal The amplitude of the *b*-wave is expressed in per cent of the pre illumination value, which in the four experimental series varied between 330 and 390 μV (*cf* Table I) The intensity of the conditioning flashes were about 7 log units (curve A and B), 8 log units (curve C) and 9 log units (curve D) above the threshold of the *b*-wave in the dark adapted eye When repetitive flashes were given (B 10 flashes, C and D, 100 flashes) the intervals were 30 sec between the flashes As shown by curve A even a single light flash with an intensity about 7 log units above the threshold of the dark adapted eye is followed by a statistically significant *reduction* of the *b*-wave The reduction lasts for about a week The conspicuous *increase* in the *b*-wave, following upon 10 flashes of the same intensity (curve B), is not statistically significant In order to find out if strong light flashes really can induce an increase of the *b*-wave, an increased flash intensity as well as an increased number of conditioning flashes were used (curve C and D) Curve C shows that 100 flashes with an intensity of about 8 log units above the *b*-wave threshold resulted in a pronounced initial increase of the *b*-wave When the intensity of the conditioning intermittent light stimulus was raised to about 9 log units above the *b*-wave threshold (D) the initial

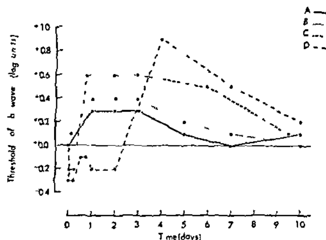


Fig 7 Threshold of *b* wave (in log units, pre illumination value referred to as zero) after illumination with single and repetitive (10 flashes in B 100 flashes in C and D) light flashes Intensity of illumination 7 (A and B), 8 (C) and 9 (D) log units above the *b* wave threshold in the dark adapted eye.

increase was even larger, lasting about 48 hours with a maximum value of 160 per cent 6 hrs *after exposure of the eye to the repetitive conditioning flashes* Following this increase a transient decrease was noted in the *b*-wave and a recovery to pre illumination values was accomplished in two weeks

In the present experiment, the influence of flashes on the *b* wave *threshold* of the dark adapted eye was also studied in the same four animals The results of this study are illustrated in Fig 7 The threshold values, expressed in log units of stimulus intensity are referred to the pre illumination value, marked zero on the vertical axis The day to-day variation of the pre illumination threshold varied within ±0 1 log units A reversible increase in the threshold corresponding to the decrease in amplitude (Fig 6) was noted in the A and B series When the intensity and number of flashes were raised (Fig 7 C and D) the increase was preceded by a decrease in threshold, corresponding to the initial increase in amplitude (Fig 6 C and D)

The results described above were all made on pigmented rabbits In additional experiments principally performed in the same way as *described in Fig 6*, similar effects were obtained on albino rabbits Thus, a pigment shielding mechanism cannot be responsible for the high intensity flash effects

As mentioned above the method described allowed both eyes to be stimulated simultaneously with light of equal intensity In order to study the effect of optic *denervation transection of the optic nerve* was made on one side, leaving the eye on the other side to serve as control

Intracranial transection of the left optic nerve of a rabbit was performed in iv Nembutal (Astra) anesthesia A craniotomy was performed *medial to the left orbit* The dura was incised and by careful suction a hole was made through the limbic cortex just inside the lateral wall of the skull cavity After exposing the optic nerve transection was performed Ophthalmoscopic control verified the integrity *of the* ocular circulation following the section The transection was confirmed by the absence of the pupillary *reaction to light on the left side*

Between 10 and 90 days after the operation similar experiments as described in Fig 6 were performed The flash effects described above were seen in both eyes although the amplitudes were 10—15 per cent higher on the left side after denervation

Discussion

The long lasting reversible effects of high intensity flashes which have been shown in the present investigation are of considerable interest since high intensity flashes have been used very often in recent experiments during the last decades First the so-called early receptor potential (ERP) (Brown and Murakami 1964) is obtained by using high stimulus intensities In a study on man for instance an intensity of "12 log units above the dark adapted threshold has been used (Galloway 1967) and in a study on the rabbit the eye was illuminated with an intensity 10 log units above the *b* wave threshold (Yonemura *et al* 1968) Secondly in clinical electroretinography the oscillatory potential is usually elicited with high intensity flashes Finally the flash effects described in the present study must be taken into account in several light perception experiments where the immediate effects after high intensity flashes were studied (Chisum 1968)

The studies on the albino rabbit and the transection experiment show that the long term effects on retinal function induced by high intensity flashes are not due to influences on a shielding pigment or to a central efferent mechanism via the optic nerve

In this context it should be mentioned that the results of studies on the effect *per se* on the *b* wave following optic denervation are somewhat controversial In the cat an increase (Jacobson and Gestring 1958 Jacobson and Suzuki 1962) as well as no effect at all (Brindley and Hamasaki 1962) have been described In the rabbit according to Abe (1962) section of the optic nerve is followed by a 330 per cent increase of the *b* wave Thus the results of the present study are more in accordance with the results in the cat reported by Jacobson and Gestring i that the optic denervation is followed by a moderate increase in ERG amplitudes

As pointed out in the introduction Dowling and Hubbard (1963) showed an initial delay in the rhodopsin formation after a brilliant flash exposure to the retina Moreover an inability of the ERG to recover in the dark following an electronic flash was described by Buckser (1966) in an experiment lasting 12 hours Probably the flash intensity in his experiment was higher than in that reported by Dowling and Hubbard and was more equivalent to that used in the present series This would mean that the reduction of the *b* wave which according to Buckser represents an inability of the ERG to recover in fact may be the initial phase of a long lasting reversible process (see curve A in Fig 6

Thus a prolonged delay in rhodopsin formation on a similar basis as the initial delay described by Dowling and Hubbard might be a possible mechanism behind the reversible long term flash effects described It must be taken into account that the

long term effect described in the present paper does not represent recovery in the dark, although all recordings were performed in dark adapted eyes, relatively dim light adaptation occurred in the intervals between the experiments

It is possible that the reversible, long lasting suppression in retinal function reported in the present work may be referred to a damaging effect Recently there have been some reports on retinal damage by continuous exposure of light The morphological damage has primarily been observed in the photo receptor outer segments (Noell et al 1966 Kuwabara and Gorn 1968 Grignolo et al 1969) The damaging effect is manifested by a slow and incomplete recovery of the ERG (Noell et al 1966) With lower intensities of the damaging light, both electroretinographical and morphological reversible changes are reported (Gorn and Kuwabara 1967 Kuwabara and Gorn 1968)

The photo receptor outer segments have been shown to undergo a constant metabolic turnover, with material migrating from the junction with the inner segment to the distal end of the outer segment (Dowling 1967 b, Young 1967) The time course of this migration varies in different species, in the rat, which in many retinal aspects is similar to the rabbit 10 days have been reported (Young 1967) A period of about this length should then be necessary for the normalization of the reversible changes due to damage to the outer segments In the present investigation the reversible changes had a duration of one or two weeks (In this context it should be mentioned that morphological studies on the flash effects described are in progress)

Post tetanic potentiation (PTP) is well known neurophysiological phenomenon which refers to an increased responsiveness following repetitive stimulation Numerous reports have described PTP in the visual system (see Hughes 1958) In the human ERG intermittent light stimulation with a frequency of 1—2/sec, has been shown to give rise to an increased responsiveness recorded immediately after the stimulation (Peregrin and Sverak 1960 1965) The initial increased responsiveness reported in Fig 6 and 7 in the present investigation might be due to a PTP mechanism although this increase remained for such a long time as 48 hours (curve D in Fig 6 and 7) and the frequency of the conditioning intermittent light was relatively low *viz* 2/min

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Properties of RNA from Giant Axons of the Crayfish

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Abstract

ANDERSSON E, A EDSTROM and J JARLSTEDT *Properties of RNA from giant axons of the crayfish* Acta physiol scand 1969 1970 *78* 491—502

The presence of axonal RNA was demonstrated in giant nerve fibres of the crayfish *Procambarus clarkii* using ultramicrochemical and radioautographic procedures The RNA content

Data are now accumulating which indicate the existence of axonal RNA within many types of neurons (Edström, Eichner and Edstrom 1962 Grampp and Edstrom 1963 Koenig 1965 b Austin and Morgan 1967 Balazs and Cocks 1967 Singer and Green 1968 Peterson Bray and Austin 1968 Hartmann Lin and Shively 1968) These findings suggest that axonal proteins are not only of perikaryonal origin delivered by axonal flow, but may also be synthesized by a local supplementary mechanism (Koenig 1965 a, Edstrom 1966 Fischer and Litvak 1967 Morgan and Austin 1968 Giuditta Dettbarn and Brzin 1968 Gordon Bensch Deanin and Gordon 1968 Edstrom and Sjostrand 1969) The information about the properties and in particular the origin of axonal RNA is still scarce mainly due to the difficulties in obtaining pure and uncontaminated axoplasm in quantities large enough for chemical analyses The ventral nerve cord of the crayfish *Procambarus clarkii* has here been used for further studies of these questions With ultramicrochemical techniques and radioautography the properties and patterns of synthesis of axonal RNA have been studied and compared to that of RNA in Schwann sheaths and ganglionic nerve cell bodies

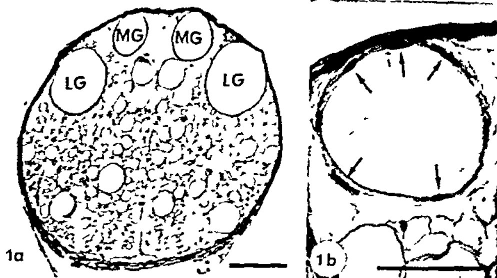


Fig. 1. Cross sections through an abdominal nerve cord of *Procambarus clarkii* between two ganglions (1 a) and through a MG fibre (1 b). MG = medial giant fibre. LG = lateral giant fibre. Arrows indicate Schwann nuclei. Scale = 100 μ.

Methods

Animals. Specimens of the crayfish *Procambarus clarkii* were used in this investigation. The crayfish was obtained from Dahl Co., Berkeley, California and transported by air to Gothenburg. They were kept in large tanks at room temperature for at least 10 days before they were used in experiments.

The giant nerve fibres. The anatomy of the giant fibre system has been repeatedly investigated and is here only briefly described. Two pairs of giant fibres are found in the ventral cord (Fig. 1 a). The medial giant (MG) fibres are situated near the midline on the dorsal side of the cord. They run uninterrupted from the brain, where they synapse with each other, to the telson. The cell bodies (up to 180 μ in diameter) lie in the procerebrum. The architecture of the MG fibres has been elucidated by Horiuchi, Hayashi and Takahashi (1966). The lateral giant (LG) fibres are separate segmental interneurons, each having one ganglionic contralateral cell body (Remler, Selverston and Kennedy 1968). The diameter of the giant fibres varies from 100 μ up to 250 μ. The giant fibre sheath is lamellated, consisting of alternating layers of cell cytoplasm and connective tissue. The Schwann cell cytoplasm and nuclei are found between the axolemma and the lamellated sheath (Hama 1961). As can be seen (Fig. 1b) Schwann nuclei are rather frequent. We mostly found 4—6 nuclei on every cross section of MG fibres and 3—5 on sections of LG fibres.

Chemicals. Aqueous solutions of H^3-uridine (24—29 c/mmole) and H^3-cytidine (25 c/mmole) were purchased from the Radiochemical Center, Amersham, England. Actinomycin D was a generous gift of Dr. C. W. Mushett, Merck Sharp and Dohme, Rahway, New Jersey, U.S.A. Two commercial preparations of ribonuclease (RNase) were used. RNase (Worthington [illegible] at a concentration of 400 μg/ml in 0.2 N [illegible] RNA analysis with J. E. Edström's method [illegible] ized (protease free) at a concentration of [illegible] aphic approach. Physiological salt solution [illegible] lium for the ventral nerve cord. It has the following composition in mM: NaCl 205, KCl 5.4, $CaCl_2$ 13.5, $MgCl_2$ 2.6, $NaHCO_3$ 2.3. One g glucose was added per litre salt solution and pH was adjusted to 7.5 by adding small amounts of HCl.

Preparation of tissue constituents for RNA analysis The ventral nerve cord was dissected from the crayfish and fixed *in toto* either in Carnoy's solution (ethanol-chloroform acetic acid 6 3 1, v/v) or in Bouin's sol
for 60—90
on cover g
ethanol 4
de Fonbrune
ated axoplasm of the medial and lateral axons
nerve cell bodies respectively could be isolated
and Schwann sheath were calculated from their
The volume of the nerve cell body was estimated from the formula
the 3 diameters determined on isolated cell
Micklewright Kurnick and Hodes 1953) In
with a tip of about 10—20 μ and filled with
nt axon and axoplasm was sucked into the
s, evaporated to dryness and washed for 10
% ethanol, prior to RNA analysis With this
pure axoplasm, free from other tissue constituents, could quickly be prepared The different tissue collections were extracted with RNase and their RNA content and base composition were determined according to Edstrom (1964)

Radioautog

transferred to Bouin's fixative for 1 hr The specimens were then passed through 70 % ethanol, 70 % ethanol tetrahydrofuran (1 1), and tetrahydrofuran before embedding Prior to radioautography deparaff ished in the cold in 5 % PCA for 1 were extracted with RNase for 120 on (Ilford K2) according to a slight nd (1962) The emulsion was melted at 40° in a water bath and then diluted with an equal part of redistilled water The slides were coated with a soft metal spatula, and placed vertically at room temperature The air-dried radioautographs were placed in taped plastic boxes containing desiccant in a refrigerator at 4° and exposed for periods varying from 20 to 45 days The radioautographs were developed in Ilford ID 19 (1 1) for 4 min, washed briefly in distilled water and fixed in 30 % sodiumthiosulphate for 8 min All procedures were made at a constant temperature of 18° After rinsing in big volumes of distilled water the slides were stained in haematoxylin (Ehrlich) and eosine or in toluidine blue

In some experiments scintillation counting was used to determine the radioactivity of whole tissue Specimens were then put in cold 10 % TCA to stop incorporation and homogenized After centrifugation the precipitate was resuspended and centrifuged in 2 ml of cold 10 % TCA The procedure was repeat washes with 95 % ethanol RN (1945) with 0 5 ml 5 % TCA washed once with 0 2 ml 5 % RNA determinations according t in 15 ml of a dioxane-PPO POPOP solution The measurements of radioactivity were made in a TriCarb liquid scintillation counter Quench correction was used for calculation of dpm In separate analyses the protein content of the abdominal nerve cord was determined by the Lowry technique (1951)

Results

RNA in giant nerve fibre compartments and in ganglionic nerve cell bodies

RNA content and concentration

The RNA concentration (w/v) of nerve cell bodies was very constant, about 5 7 %, in spite of considerable variations in their cell volumes (Table I) The axonal RNA concentration averaged 0 02 % No remarkable variations in the axonal RNA concentration were observed between giant fibres of different size (100—200 μ across)

TABLE I. RNA content and concentration in nerve cell bodies, axons and Schwann sheaths of giant nerve fibres from the crayfish

Tissue constituent	RNA content in μμg	n*	N**	% RNA (W/V) ± S.E.M.	n*	N**
Nerve cell bodies	300—600 (mean 500)	18	6	5.7 ± 0.3	10	3
Axons	1—2 (mean 1.67/μ axon)	9	6	0.02 ± 0.002	5	3
Schwann sheaths	1.9—2.5 (mean 2.2/μ sheath)	5	3	0.22 ± 0.01	5	3

* n represents the number of determinations

** N represents the number of animals

Analyses have been performed on isolated nerve cell bodies from abdominal nerve cord ganglia, isolated 200—350 μ long segments of giant axons, and Schwann sheaths where each sample represents the sheath surrounding a 1120 μ long axon segment

from different levels of the abdominal nerve cord. Analyses of the MG and LG fibres were performed at distances of 2 to 4 cm (MG fibres) and 0.2 to 0.4 cm (LG fibres) from their perikaryonal origin. The concentration of RNA in the MG axon was similar to that of the LG axon. It therefore seems as if the axonal RNA concentration in the giant fibres is not markedly related to the distance from the cell body. Small changes in RNA concentration along the course of the axons cannot be excluded, however, before this possibility has been more systematically tested. As can be seen from Table I the Schwann sheath RNA concentration exceeds that of the axon about ten times, but due to volume differences, the total amount of RNA per unit length fibre is about the same for both compartments.

RNA base composition

The base composition of RNA from axons and nerve cell bodies were quite different with dissimilarities both in purines and pyrimidines (Table II). Most of the RNA in nerve cell bodies is cytoplasmic and therefore likely to be ribosomal. The quotient

TABLE II. Base composition of RNA from axons and Schwann sheaths of giant nerve fibres and from ganglionic nerve cell bodies in the crayfish. Base ratios are expressed as molar proportions in per cent of the sum ± S.E.M.

Tissue constituent	Adenine	Guanine	Cytosine	Uracil	G+C/A+U	n*
Nerve cell bodies	24.4 ± 2.0	22.5 ± 1.0	26.1 ± 3.2	27.0 ± 1.8	0.97 ± 0.10	4
Axons	30.2 ± 3.1	21.7 ± 0.3	13.6 ± 2.3	34.6 ± 1.8	0.53 ± 0.04	5
Schwann sheaths	28.1 ± 0.8	27.4 ± 2.2	15.5 ± 3.0	29.1 ± 1.9	0.77 ± 0.05	5

* n represents the number of hydrolyses performed on separate animals. Each hydrolysis value is the mean of two to four microelectrophoretic separations

P value for the significance of the difference in G + C/A + U quotients between axons and nerve cell bodies: $P < 0.01$

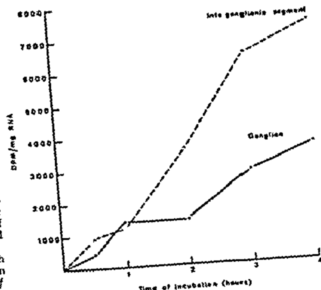


Fig 2. Time course of incorporation of H^3 uridine into RNA of ganglionic and interganglionic parts of abdominal nerve cords (cf Results) Nerve cords were incubated for varying periods of time at 18° in 500 µl Harreveld ...tion containing 10 µc H^3 ...ine (24 0 c/mmole) Each ...nt in the curves is based on ...lysis of two samples (cf ...thods)

+C/A+U, was 0 97 for this RNA The axonal RNA on the other hand shows a ...ore DNA like base composition with a lower quotient 0 53 This indicates that the ...lative content of ribosomal RNA is small in the giant axons The Schwann sheath ...NA with a quotient of 0 77 occupies an intermediate position possibly because its ...lative content of cytoplasmic RNA is likely to be smaller than for nerve cell bodies ...nalyses of fresh axoplasm prepared by the micropipette technique from MG fibres ...ielded four clear peaks upon microelectrophoresis Due to a high protein contamina...tion of the pyrimidines the molar base proportions are not in agreement with those of RNA from fixed and dissected axoplasm To investigate the possibility that mito-chondria were the source of axonal RNA a sample of fresh axoplasm representing a volume known to contain at least 1000 µµg RNA was placed on an electron micro-scopic grid When the drop after about 20 sec had evaporated to about 50—75 % of its initial volume it was washed away with destilled water It is known that bio-logical structures stick firmly to the supporting film contacting its surface during the sedimentation and therefore will not be washed away (cf Sjostrand 1967) Examina-tion of 75 % of the grid area covered by the specimen stained with uranyl acetate disclosed only 4—6 structures identified as mitochondria It is consequently likely that the bulk of axonal RNA is localized outside mitochondria

Linearity of incorporation

Abdominal nerve cords were incubated for 0 5 1 2 3 and 4 hrs in Harreveld solu-tion containing tritiated uridine After incubation each cord was separated into two samples one of which consisted of 5 ganglions and the other one of the remaining interganglionic parts RNA was determined and the radioactivity plotted (Fig 2) It



Fig 3 Radioautographs of giant fibres after incubation of an abdominal nerve cord at 18° 3 hrs in one ml Harreveld solution containing 25 μc H³ uridine (29 0 c/mmole) Cross secti of a LG fibre (3a) and a MG fibre (3b) (Ax) axoplasm (S s) Schwann sheath (E) em space (fixation artifact) showing grain concentration over background Arrows ind Schwann nuclei Exposure time = 45 days Scale = 100 μ (phase-contrast microscopy)

can be seen that the incorporation is largely linear for a period of 4 hrs The experi ment indicates that the *in vitro* system is suitable for incorporation studies and also agrees with experiments showing unchanged action and membrane potential the MG fibres for at least 12 hrs during similar incubation conditions (Edstrom an Hansson unpublished) The incorporation rate was found to be about twice as hi in the interganglionic as in the ganglionic samples Since there are no blood vess inside the cord the incorporation in the interganglionic segments can largely be attri buted to Schwann sheaths and axons Besides these elements about one third of th volume of the ganglia is occupied by nerve cell bodies and glial capsules In separat experiments based on analyses of 5 abdominal nerve cords it was also found that th RNA concentration (μg RNA/mg protein) measured for ganglionic samples 25 2± 5 0 (mean ± S D) was higher than that for interganglionic samples 13 2 ± 2 3 Th higher RNA concentration in the ganglionic samples is likely to be due mainly to the presence of nerve cell bodies The nerve cell body is from other, mainly *in vi* studies recognized as the dominating RNA producer among nervous elements Th difference in incorporation rate was therefore surprising and suggests that a consider able part of ganglionic RNA is rather stable as compared to nerve fibre RNA Th results are not conclusive however since the existence of penetration barriers of varying efficiency for the radioactive precursor in the ganglionic and interganglionic parts might influence the results It is interesting however that in the Mauthner neuron axonal RNA labels considerably more rapidly than RNA from the whole spinal cord (Edstrom Edstrom and Hokfelt 1969)









Fig 4 Radioautographs of MG fibres (phase-contrast microscopy of longitudinal sections) and ganglionic nerve cell bodies (light microscopy) after incubation of abdominal nerve cord for 5 hrs at 18° in one ml Harreveld solution containing 5 μc H^3 uridine (24 0 c/mmole) and 5 μc H^3-cytidine (25 0 c/mmole) with (4c 4d) and without actinomycin D (4a 4b) Nerve cell bodies and Schwann nuclei are indicated by short and long arrows respectively (Ax) axoplasm (S s) Schwann sheath Exposure time—20 days Scale—60 μ (4a 4c) Scale—30 μ (4b, 4d)

Radioautography

Abdominal nerve cords were incubated for varying times in Harreveld salt solution containing tritiated uridine The Schwann nuclei of both the LG and MG fibres were weakly labelled after incubation for 0 5 hr and the grain concentration over the axoplasm only slightly exceeded the background At 5 hrs the nuclei were intensely labelled and a significant activity was obtained in the axoplasm The number of grains counted per unit area over the Schwann sheath was about intermediate to that found over nuclei and axoplasm The same distribution of activity was found in the MG and LG fibres (Fig 3) In another experiment it was shown that incorporation of tritiated uridine and cytidine was almost completely removed by RNase The incorporation also showed sensitivity to actinomycin D (Fig 4) However, at a concentration (10 μg/ml) which reduced the RNA incorporated activity in ganglionic nerve cell bodies by about 70 %, the percentage inhibition measured over the different giant fibre compartments was only 40—50 The difference in sensitivity might be due to the existence of a penetration barrier more effective towards actinomycin D in the nerve fibre than in the nerve cell bodies It is also possible that some unspecific labelling, not related to RNA synthesis could have occurred The effects caused by RNase and actinomycin D indicate that the incorporated activity at least in part is due to newly synthesized RNA

Frequently, the histological procedures were found to cause displacement and shrinkage of the axoplasm, but on several of the best preserved preparations accumulations or gradients of grains were observed over the axoplasm in its exterior part and sometimes preferably in its vicinity to Schwann nuclei (Fig 5) It is unlikely that these grain concentrations are caused by redistribution or leakage of incorporated activity during the histological procedures, since radioautographic blackening was obtained over the axoplasm and the grains were always evenly scattered when a protein precursor (H^3-leucine) was used The most likely explanation for the finding of activity close to the axolemma and the Schwann nuclei is that RNA synthesized in these nuclei is transferred via Schwann cell cytoplasm to the axon compartment It cannot however, be excluded that the RNA precursor as such is transported via a similar route and becomes available for axonal RNA synthesis by local mechanisms

Discussion

This study adds further support to previous now numerous observations of the existence of RNA in nerve axons The presence of RNA within the axoplasm of crayfish giant nerve fibres could be ascertained by two different sampling procedures both of which have the advantage that a layer of axoplasm adjoining to the Schwann sheath can be avoided thereby eliminating extraneous tissue as a source of RNA The volume of axoplasm which is not included in the axon samples represent for the fixed and dissected axons a negligible part of the total volume Base determinations on fresh axonal RNA did not show the same reproducibility as those on fixed and



Fig 5. Radioautograph... hrs with 25 μc H³-... (5a) and MG (5b, 5... nuclei Exposure ti... microscopy

dissected axoplasm. This may be due to difficulties in obtaining series of identical samples with the use of micropipettes. Furthermore, RNA hydrolysates prepared from fresh axoplasm were considerably contaminated with proteins RNA analyses on fixed and dissected axons were therefore used for comparisons with RNA from nerve cell bodies and Schwann sheaths In view of the low RNA concentration found in earlier studies of axons, as compared to that of cell bodies and Schwann sheaths, it could possibly have been argued that the RNA found in the microdissected axon samples could have been redistributed from other tissue components during the penetration of the fixatives in the nerve cord This objection can now be ruled out since RNA was also found in fresh axoplasm collected by micropipettes. These findings support the validity of previous RNA determinations performed on fixed and microdissected materials from other systems (Edstrom *et al.* 1962, Grampp and Edström 1963, Koenig 1965b, Hartmann, Lin and Shively 1968) The axoplasm contains about 1 67 μμg RNA per μ axon length, which is valid for both LG and MG axons in the abdominal region of the ventral cord. A 4 cm long MG axon is thus likely to contain at least 67,000 μμg RNA, but probably much more, since the axon increases its diameter in the rostral region with up to 100 %. The RNA cor

tent of its corresponding cell body is unknown, but is not likely to exceed 1000 μμg RNA a value not even attained by the biggest ganglionic nerve cell bodies analysed having cell volumes comparable to that of the MG neuron It is obvious that in the MG neuron the total amount of axonal RNA, in spite of its low concentration (0 02 %), makes up the major part of neuronal RNA In this respect the MG neuron is not an exclusive example A similar axonal RNA concentration (0 03–0 07 %) has been described for the Mauthner neuron of goldfish (Edstrom 1964) in which also the bulk of RNA was found to be axonal The concentration of RNA in axonal balloons (0 23 %) from the spinal cord of rat was recently determined (Hartmann Lin and Shively 1968) and found higher than the values given above for normal axons It should, however, be observed that concentration values must be considered relative since fixation always causes a certain reduction in volume

There is now evidence for protein synthesis in the axon in several species (cf Introduction) In the present case this synthesis might be quite considerable compared to that of the cell body considering the intracellular distribution of RNA

Tritiated uridine and cytidine are incorporated into the axoplasm of the MG and LG neurons in a process sensitive to actinomycin D, wellknown to inhibit DNA dependent RNA synthesis (Wintersberger 1966) The incorporated label shows resistance to cold PCA but is extracted by RNase According to these criteria the labelled substance is newly synthesized RNA In the incubation system used the MG axons are separated from their perikarya in contrast to the LG axons which are parts of integrated units In the MG axons the incorporation thus occurs in a process independent of the perikarya We do not exclude that axonal RNA is normally in part synthesized in the perikaryon and transported by axonal flow A perikaryonal origin of RNA in other systems has been suggested by Bray and Austin (1968) Peterson Bray and Austin (1968) and Casola, Davis and Davis (1969) but this still remains to be proved We found about the same relative incorporation in the axoplasm of both LG and MG axons which makes it likely that contribution of RNA by axonal flow from perikarya is negligible during the time of incubation used or does not exist in the LG axons The pronounced difference in base composition between axonal RNA and RNA of nerve cell bodies is also in favour of a local rather than central origin of axonal RNA

Mitochondria are so far the only extranuclear organelles known to synthesize RNA in the nervous system The axonal RNA might therefore have a mitochondrial origin It is however probable that the bulk of axonal RNA is localized outside the mitochondria since samples of axoplasm prepared by the micropipette technique consisting of the central core of the axons contain significant amounts of RNA but very few mitochondria according to electron microscopic analysis Most mitochondria are concentrated in a layer close to the axon Schwann interface (Hama 1961) and are therefore not included in the samples There is another argument in support of an extramitochondrial localization of axonal RNA The protein concentration of crayfish axoplasm is unknown If however the protein concentration is about the same as has been measured for squid axoplasm (3–4 %) by

Bear and Schmitt (1939), the ratio RNA/protein can be estimated for crayfish giant axons and is approximately 0 006 (w/w) Data presented by Truman and Korner (1962) and by Balazs and Cocks (1967) show that values for purified mitochondrial fractions from organs as different as liver and brain all lie within the narrow range 0 007—0 010 The value calculated for crayfish axoplasm is of the same order of magnitude Mitochondria can only account for a quantitatively negligible part of the total protein content of crayfish giant axons and it is thus obvious that the bulk of crayfish axonal RNA has an extramitochondrial localization. This is valid even if the ratio calculated for crayfish axoplasm is a five fold overestimation, due to our lack of knowledge of the true protein concentration in the axoplasm It cannot be excluded that axonal RNA is synthesized in the mitochondria and exported from the mitochondria Of relevance for this possibility is the finding in a previous work (Edstrom *et al* 1969) that axonal RNA of Mauthner neurons was synthesized independent of any nuclei However, results were also obtained suggesting that Mauthner axonal RNA could partly have an origin in surrounding tissues Singer and Green (1968) proposed on basis of radioautographic studies that RNA in peripheral nerve axons of the newt may be derived from Schwann nuclei Some findings in this work are also in accordance with this suggestion The observation of accumulations and gradients of RNA label in the axon in its vicinity to the Schwann nuclei, suggest that the RNA source might be Schwann nuclei Schwann nuclei are relatively abundant in the sheaths of the giant axons Three to six nuclei are mostly seen in each cross section of the fibres and they are located between the myelin and the axon in contrast to the case in vertebrates where Schwann nuclei are found outside the myelin The cytoarchitecture of crayfish giant axons could therefore facilitate an RNA exchange between the Schwann cell and the axon Although Singer and Green (1968) have recently suggested an intercellular RNA transfer in the nervous system there is still no direct proof for its existence

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Interactions between Inhibitors of the Axonal Amine Pump and Bretylium in the Degeneration Release of Sympathetic Transmitter

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Abstract

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The time course of the degeneration contraction of periorbital smooth muscles has been studied in conscious rats given different inhibitors of the axonal amine pump either alone or in combination with bretylium. Desmethylimipramine (DMI) or cocaine at 20 mg/kg did not influence the time of onset of the degeneration contraction while Lu 3-010 (10 mg/kg) caused a somewhat premature start. Lu 3-010, DMI or protriptyline at 10 mg/kg but not trimipramine or cocaine at 20 mg/kg antagonized bretylium. They reduced and prevented (DMI) the bretylium induced delay DMI reduced the delay by a non-competitive mechanism. The antagonism to bretylium does not seem to be explainable by simple interference with the axonal amine pump Indirect evidence suggests that the duration of the delay induced by bretylium is related to the rate of disappearance of bretylium from critical sites in the nerve endings — DMI and Lu 3-010 seem to have somewhat different actions on the degenerating adrenergic nerve terminals

During the first few days after denervation the nerve endings in sympathetically innervated organs loose their transmitter content (Kirpekar *et al* 1962 Weiner *et al* 1962, Benmiloud and Euler 1963 Malmfors and Sachs 1965) From a functional study recently published (Lundberg 1969a) it was reported that in the rat starting 14 to 15 hrs after excision of the superior cervical ganglion there was a spontaneous and transient reversal of the postdenervation ptosis and enopthalmus lasting for about 12 hrs This denervation phenomenon was considered to be a degeneration contraction of periorbital smooth muscles and caused by release of stored noradrenaline from the degenerating nerve terminals in accordance with the transmitter release hypothesis (Fleckenstein and Burn 1953 Emmelin and Stromblad 1957 Sears and Barany 1960) The finding in earlier studies with other methods (Benmiloud and Euler 1963 Malmfors and Sachs 1965 Lang— 1966) that

ergic neuron blocking agents could induce a delay of the postdenervation loss of sympathetic transmitter was fully confirmed in the study of the degeneration contraction in the rat It was also shown that of the 4 potent neuron blockers used bretylium had a superior delaying effect

The present paper reports further studies on the pharmacology of the degeneration contraction in the rat It deals especially with the ability of different inhibitors of the axonal amine pump to antagonize the delaying effect of bretylium

Materials and Methods

For details, see Lundberg 1969a

Experimental animals

Male Sprague Dawley rats weighing between 200 and 300 g and kept in normal day light conditions at around 23° C were used Commercial rat food pellets (No 210 Anticimex Sollentuna, Sweden) and tap water were provided *ad lib* In some experiments the rectal temperatur was checked during the period of treatment with a thermistor probe In no case the body temperature deviated more than 1° C from the pre treatment values

Surgical procedure

With the aid of a Zeiss operation microscope and under ether anesthesia the right superior cervical ganglion was extirpated (denervation) On the left side the pre ganglionic trunk was cut (decentralisation)

Drugs

The following drugs were
Green the Wellcome Res
chloride (Pertofran®) (G
(Merck Sharp & Dohme,
maleate (Surmontil®) (L
inopropyl)
ntle warmin
ts All inj
tions were subcutaneous

Measurement procedure

The sizes of the palpebral apertures of the two eyes in conscious animals were estimated almost simultaneously at a distance A special apparatus with an image splitting eye piece as the principal part was used For graphical representation of the degeneration contraction the differences in mean size of aperture between the denervated and the decentralized (control) side at every occasion of measurement were calculated and plotted against the times after denervation Curves of individual animals thus obtained were then analyzed as earlier described (Lundberg 1969a) The degeneration contraction was characterized from the following points of view

The time course The times corresponding to 50 and 75 per cent of maximal difference between denervated and decentralized side on the ascending part (T_{50a} and T_{75a}) and on the descending part (T_{50d} and T_{75d}) of the curves were of especial interest

The duration This was measured as the difference between T_{50d} and T_{50a} (the width)

The slopes of the curve limbs The difference between T_{75a} and T_{50a} (rise time) and T_{50d} and T_{75d} (fall time) is an inverse expression for the slopes of the ascending and the descending limbs respectively

The height This is the maximal value of palpebral aperture measured on the denervated side during the degeneration contraction no regard being taken to the control side

The values of interest are then pooled and treated statistically *Student's t* test was used for the analysis of significance

Results

Effects of desmethylimipramine (DMI), Lu 3-010 and cocaine given alone on the degeneration contraction

Rats were treated with DMI, Lu 3 010 or cocaine within 5 hrs before the expected start of the denervation phenomenon The control animals run simultaneously were given 0 9 per cent sodium chloride instead of the tested drug The design of treatment and the results are shown in Table I The time of start of the degeneration contraction measured as T_{50_a} was not markedly influenced by cocaine or DMI even at 20 mg/kg However Lu 3 010 started the contraction somewhat prematurely In all treated groups except in the cocaine group the drugs showed a tendency to shorten the duration and with the exception of one DMI group to increase the slope of the descending curve limb Whether this is a constant effect is doubtful The sizes of the palpebral apertures especially that of the decentralized side tended to be increased by the drugs The aperture on the decentralized side started to increase within 1 hr after the injection of the drug and with DMI or Lu 3 010 it was quite stable for at least 10 to 12 hrs and did not change while the degeneration contraction was proceeding on the other side With cocaine the increase of aperture on the decentralized side did not last for more than 5 to 6 hrs This effect on the control side thus made it possible to check the duration of the drug Since the size of the aperture on the decentralized (control) side increased comparatively more than that on the denervated side and since the values of the difference between the two sides were used for construction of the contraction curve (see Methods) the contraction curve was apparently lowered and shortened Owing to the fast appearance and disappearance of the degeneration contraction the slopes of the limbs of the contraction curve are steep and the influence of the control side on T_{50_a} and T_{50_d} is small Similar effects on the size of the palpebral aperture on the decentralized side were seen even in the experiments reported later where DMI protriptyline and Lu 3 010 were given in combination with bretylium see Table II and III

Inhibitors of the axonal amine pump and the bretylium induced delay of the degeneration contraction

Rats were given bretylium 11 hrs after ganglionectomy and the drugs tested 2 hrs later which is about 2 hrs before the time when the degeneration contraction normally is expected to start. The control animals also were given bretylium at 11 hrs but 0 9 per cent sodium chloride instead of the tested drug The effects of desmethylimipramine (DMI) protriptyline trimipramine Lu 3 010 and cocaine were thus examined All these drugs except trimipramine are known to have an inhibitory action on the uptake of amines at the level of the axon membrane (for references see Discussion) The results are shown in Fig 1 and Table II The drugs which are potent inhibitors of the axonal pump mechanism seem to be very active ones in reducing the bretylium induced delay Cocaine however had no such effect This lack of effect of cocaine was tested using a higher dose given at the time when the

TABLE I Effects of DMI, Lu 3 010 and of cocaine given alone on the degeneration contraction The injections were subcutaneous The control rats run simultaneously were given 0 9 % NaCl instead of tested drug T_{50a} and T_{75a} are the times of 50 and 75 per cent of maximum development of the degeneration contraction T_{50d} and T_{75d} are the corresponding levels on the descending phase The values are M±S E M The values of the experimental groups have been compared to those of the corresponding control groups The significance levels are *** = p < 0 001, ** = p < 0 01, * = p < 0 05

Treatment design	n	Time of start (T_{50a}) hours[2]	Duration (T_{50d}—T_{50a}) hours	Height (peak aperture on denervated side) mm	Aperture on decentralized side[1] mm	Rise time (T_{75a}—T_{50a}) hours	Fall time (T_{50d}—T_{75d}) hours
DMI 20 mg/kg at 10 hrs[2]	5	14 99±0 63	6 05±1 18	4 74±0 17	3 23±0 20	1 29±0 41	1 24±0 55
Saline	4	14 53±0 40	7 04±0 69	4 89±0 12	2 82±0 16	1 31±0 36	1 76±0 34
DMI 15 mg/kg at 12 hrs	5	14 82±0 25	7 55±0 20	5 11±0 07*	3 73±0 15**	0 90±0 19	2 83±0 59
Saline	5	14 76±0 21	8 10±0 40	4 52±0 18	2 98±0 08	0 59±0 07	2 59±0 37
DMI 10 mg/kg at 13 hrs	5	15 12±0 39	6 28±0 21*	4 58±0 12	3 49±0 10*	0 65±0 10	1 13±0 19
Saline	6	14 68±0 17	8 79±0 92	4 57±0 07	3 20±0 07	0 63±0 11	1 83±0 32
Lu 3-010 10 mg/kg at 11 hrs	6	13 76±0 36*	6 92±0 41	4 75±0 89**	3 32±0 17**	1 08±0 22	1 57±0 39
Cocaine 20 mg/kg at 12 hrs	5	14 72±0 25	9 39±0 48	4 48±0 13	3 12±0 13*	1 32±0 38	3 29±0 51
Saline	5	14 96±0 16	7 97±0 55	4 03±0 20	2 37±0 19	0 94±0 30	2 01±0 48

[1] the size of palpebral aperture on the decentralized (control) side when the degeneration contraction had its peak on the denervated side

[2] Time of start and time of injection are expressed in hours after denervation



Saline	ml/kg	2	—	—	—	—	—	—
Bretylium	mg/kg	—	4	4	4	4	4	4
Trimipramine	,,	—	—	10	—	—	—	—
Cocaine	,,	—	—	—	10	—	—	—
Protriptyline	,,	—	—	—	—	10	—	—
DMI	,,	—	—	—	—	—	10	—
Lu 3-010	,,	—	—	—	—	—	—	10

Fig 1 Effect of different inhibitors of the axonal amine pump and of trimipramine on the bretylium induced delay of the degeneration contraction All drugs were given subcutaneously, bretylium at 11 hrs after denervation and the tested drugs 2 hrs later T_{50a} is the time of half maximum development of the degeneration contraction The filled parts of the bars represent the delays of the groups The group of untreated rats (n=49) had T_{50a}=15 17±0 11 hrs (mean±s e m) There were 5 rats in each experimental group

normal phenomenon would have started Three rats were given 4 mg/kg bretylium at 12 hrs and 20 mg/kg cocaine at 15 hrs after denervation, respectively The T_{50a} was 19 24±0 38 (mean and s e m) Thus again the bretylium-induced delay was apparently not reduced by cocaine

In some experiments the drug tested for blocking effect was given before bretylium Bretylium was always injected at 13 hrs and the blockers at 11 hrs with the exception of trimipramine and two cocaine groups The results are shown in Fig 2 and Table III DMI at 10 mg/kg almost completely inhibited the delaying effect of bretylium Trimipramine and cocaine at the same doses and cocaine at 20 mg/kg had no preventing effect In fact cocaine at 20 mg/kg even significantly increased the delay

The reducing effect of DMI on the delay induced by different doses of bretylium

Bretylium at doses ranging from 0 04 to 30 0 mg per kg was given 11 hrs after denervation to rats in groups of 5 Two hrs later the animals received DMI at 10 mg/kg or 0 9 per cent sodium chloride (control groups) The results are shown in Fig 3 The dose response curve of bretylium is steep 0 08 mg/kg of bretylium has virtually no delaying action while 0 5 mg/kg has an almost full effect The shortening in hrs of the bretylium induced delay caused by DMI was not less when bretylium was given at 30 mg/kg than at 0 5 mg/kg This indicates that the antagonism is of non competitive nature

Neither bretylium at 0 04 and 0 08 mg/kg nor 10 mg/kg of DMI given alone

TABLE II Effect of different inhibitors of the axonal amine pump and of trimipramine on the bretylium induced delay of the degeneration contraction All drugs were given subcutaneously, bretylium (4 mg/kg) at 11 hrs after denervation and the tested drugs (10 mg/kg) 2 hrs later T_{50a} is the time of half maximum development of degeneration contraction T_{50d} is the corresponding level on the descending phase The values are M ± S E M The values of the experimental groups have been compared with those of two control groups Significances indicated by * refer to comparison with bretylium NaCl, those indicated by refer + to comparison with untreated rats The significance levels are ***/+++ = p < 0 001, **/++ = p < 0 01, */+ = p < 0 05

Inhibiting drug	n	Time of start (T_{50a}) hours[2]	Duration (T_{50d}—T_{50a}) hours	Height (peak aperture on denervated side) mm	Aperture on de centralized side[1] mm
Saline	10	+++ 20 31 ± 0 25	7 43 ± 0 42	4 53 ± 0 14	++ 2 74 ± 0 13
Trimipramine	5	+++ 20 91 ± 0 56	7 62 ± 0 55	4 66 ± 0 12	+ 2 78 ± 0 08
Cocaine	5	+++ 20 65 ± 0 27	7 49 ± 0 15	* ++ 4 99 ± 0 07	2 88 - 0 15
Protriptyline	5	*** +++ 16 71 ± 0 06	+ 6 94 ± 0 47	** +++ 5 29 ± 0 07	*** +++ 3 86 ± 0 13
DMI	5	*** +++ 16 43 ± 0 28	7 57 ± 0 32	** +++ 5 20 ± 0 05	*** +++ 3 77 ± 0 16
Lu 3 010	5	*** +++ 15 82 ± 0 17	7 15 ± 0 49	** +++ 5 25 ± 0 01	*** +++ 3 74 _ 0 08
Untreated rats	16	14 64 + 0 12	8 34 ± 0 30	4 62 ± 0 07	3 16 ± 0 07

[1] the size of aperture on the decentralized (control) side when the degeneration contraction had its peak on the denervated side

[2] hours after denervation

influence the start of the degeneration contraction, see Fig 3 and Table I However, the combinations of bretylium at very low doses with DMI at 10 mg/kg seemed to start the contraction even earlier than normally see Table IV An attempt to increase this effect was unsuccessful, since when bretylium and DMI were injected 1 hr earlier than done before i e at 10 and 12 hrs respectively the degeneration contraction did not start significantly earlier than normally For more reliable evaluation of the effect of low doses of bretylium in combination with DMI, however, further investigation is needed



Saline	ml/kg	2	—	—	—	—	—	—
Bretylium	mg/kg	—	4	4	4	4	4	4
Cocaine	,,	—	—	10	—	—	—	—
Cocaine*	,,	—	—	—	10	—	—	—
Cocaine*	,,	—	—	—	—	20	—	—
Trimipramine*	,,	—	—	—	—	—	10	—
DMI	,,	—	—	—	—	—	—	10

* given 12 5 hrs after denervation

Fig 2 Effect of pre treatment with DMI, cocaine or trimipramine on the bretylium induced delay of the degeneration contraction All drugs were given subcutaneously the tested drugs at 11 hrs if not otherwise stated and bretylium always at 13 hrs after denervation T_{50a} is the time of half maximum development of the degeneration contraction The filled parts of the bars represent the delays of the groups The group of untreated rats ($n=49$) had T_{50a} 15 17 ± 0 11 (mean ± s e m) There were 5 rats in each experimental group

The reducing effect of 20 mg/kg DMI injected at 15 hrs after denervation was studied in a group of 5 rats, which were treated with 4 mg/kg of bretylium at 13 hrs The increased dose of DMI had a larger effect The bretylium induced delay of this group was somewhat shorter than that of the corresponding 13—15 hrs group with 10 mg/kg of DMI The mean T_{50a} of the groups were 17 85 ± 0 33 and 18 78 ± 0 14 (mean and s e m), respectively, the difference in T_{50a} being probably significant ($p < 0$ 05)

Influence of the time intervals between the injections of bretylium and DMI or Lu 3 010 and between the ganglionectomy and the injections

Rats in groups of 5 or 10 were treated with bretylium at 4 mg/kg and DMI or Lu 3 010 at 10 mg/kg Bretylium was given at the latest 2 hrs before the blocking drug The drugs were injected at different times after denervation The control animals received bretylium only The schedule of treatment is shown in Table V and VI

Experiments with DMI

For definitions of the variables used, see Fig 4 and 5 and the legend to Table V

In 3 groups the times of bretylium injection were fixed at 11 hrs but those of DMI injection varied see Fig 4 and Table V DMI reduced the delay in all groups but there always remained a delay of varying length The shorter delay in the 11—13 hrs group compared to

TABLE III Effect of pre treatment with DMI, cocaine or trimipramine on the bretylium induced delay of the degeneration contraction All drugs were given subcutaneously, the tested drugs (10 mg/kg) at 11 hrs, if not otherwise stated and bretylium (4 mg kg) always at 13 hrs after denervation T_{50a} is the time of half maximum development of degeneration contraction T_{50d} is the corresponding level on the descending phase The values are M±S E M The values of the experimental groups have been compared with those of two control groups Significances indicated by * refer to comparison with bretylium NaCl those indicated by + refer to comparison with untreated rats The significance levels are $\overset{***}{+++} = p < 0.001$, $\overset{**}{++} = p < 0.01$, $\overset{*}{+} = p < 0.05$

Inhibiting drug	n	Time of start (T_{50a}) hours[2]	Duration ($T_{50d}-T_{50a}$) hours	Height (peak aperture on denervated side) mm	Aperture on decentralized side[1] mm
Saline	10	+++ 20 51±0 33	not read	4 75±0 11	3 23±0 13
Cocaine	5	+++ 20 70±0 59	,,	4 33±0 21	2 90±0 30
Cocaine (injected at 12 5 hrs after denervation)	5	+++ 21 69±0 47	,	4 53±0 12	** +++ 2 55±0 13
Cocaine 20 mg/kg (injected at 12 5 hrs after denervation)	5	*** +++ 23 45±0 44	,	4 55±0 16	3 39±0 16
Trimipramine (injected at 12 5 hrs after denervation)	2	21 80 22 20	,,	4 70 4 28	2 30 2 30
DMI	5	*** +++ 15 57±0 28	8 20±0 38	++ 5 10±0 10	+ 3 52±0 18
Untreated rats	16	14 64±0 12	8 34±0 30	4 62±0 07	3 16±0 07

[1] the size of palpebral aperture on the decentralized (control) side when the degeneration contraction had its peak on the denervated side

[2] hours after denervation

DMI and bretylium already before the start of a normal degeneration contraction DMI given alone at 13 hrs has no effect on the time of start of the contraction see Table I In the 11—17 hrs group, where DMI was given as late as 6 hrs after bretylium, the latency between the injection of DMI and the T_{50a} of the group was short In the 11—15 hrs group the latency was longer and in the 11—13 hrs group the latency (here perhaps not quite comparable) was even longer Thus, the later DMI was given the faster the degeneration contraction appeared after the injection of DMI

If the time interval between the injections of bretylium and DMI was fixed to 2 hrs but the times of injections in relation to denervation were varied see Fig 5

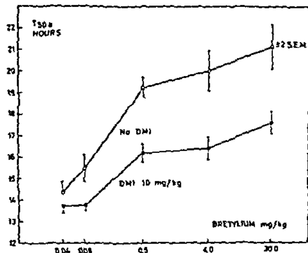


Fig 3 Reducing effect of DMI on the delay induced by different doses of bretylium. The drugs were given subcutaneously, bretylium at 11 hrs and DMI (or 0.9 % NaCl) 13 hrs after denervation T_{50a} is the time of half maximum development of the degeneration contraction There were 5 rats in each group

TABLE II Effect of the combination of a small dose of bretylium (0 04 or 0 08 mg/kg) and a moderate dose of DMI (10 mg/kg) on the time of start of the degeneration contraction The drugs were given subcutaneously bretylium at 11 hrs and DMI at 13 hrs after denervation if not otherwise stated T_{50a} is the time of half maximum development of the degeneration contraction The values are M ± S.E.M. The significance levels are *** = $p < 0.001$ ** = $p < 0.01$ * $p < 0.05$ comparisons are with the control group at the bottom of the table

Schedule of treatment	n	Time of start (T_{50a}) hours after denervation
Bretylium 0 08 mg/kg and DMI	5	** 13 76 ± 0 12
Bretylium 0 04 mg/kg and DMI	5	*** 13 73 ± 0 16
Bretylium 0 08 mg/kg and DMI (at 10 and 12 hrs after denervation respectively)	6	14 46 ± 0 32
Bretylium 0 04 mg/kg and saline	5	14 37 ± 0 35
DMI alone	5	15 12 ± 0.
Untreated rats run at the same or almost the same time	16	14 64

TABLE III Effect of pre treatment with DMI, cocaine or trimipramine on the bretylium-induced delay of the degeneration contraction All drugs were given subcutaneously the tested drugs (10 mg/kg) at 11 hrs, if not otherwise stated, and bretylium (4 mg kg) always at 13 hrs after denervation T_{50a} is the time of half maximum development of degeneration contraction T_{50d} is the corresponding level on the descending phase The values are M ± S E M The values of the experimental groups have been compared with those of two control groups Significances indicated by * refer to comparison with bretylium NaCl, those indicated by + refer to comparison with untreated rats The significance levels are ***/+++ = p < 0 001, **/++ = p < 0 01, */+ = p < 0 05

Inhibiting drug	n	Time of start (T_{50a}) hours[2]	Duration (T_{50d}—T_{50a}) hours	Height (peak aperture on denervated side) mm	Aperture on decentralized side[1] mm
Saline	10	+++ 20 51 ± 0 33	not read	4 75 ± 0 11	3 23 ± 0 13
Cocaine	5	+++ 20 70 ± 0 59	„	4 33 ± 0 21	2 90 ± 0 30
Cocaine (injected at 12 5 hrs after denervation)	5	+++ 21 69 ± 0 47	„	4 53 ± 0 12	**/+++ 2 55 ± 0 13
Cocaine 20 mg/kg (injected at 12 5 hrs after denervation)	5	***/+++ 23 45 ± 0 44	„	4 55 ± 0 16	3 39 ± 0 16
Trimipramine (injected at 12 5 hrs after denervation)	2	21 80 22 20	„	4 70 4 28	2 30 2 30
DMI	5	***/+++ 15 57 ± 0 28	8 20 ± 0 38	++ 5 10 ± 0 10	+ 3 52 ± 0 18
Untreated rats	16	14 64 ± 0 12	8 34 ± 0 30	4 62 ± 0 07	3 16 ± 0 07

[1] the size of palpebral aperture on the decentralized (control) side when the degeneration contraction had its peak on the denervated side
[2] hours after denervation

DMI and bretylium already before the start of a normal degeneration contraction DMI given alone at 13 hrs has no effect on the time of start of the contraction see Table I In the 11—17 hrs group, where DMI was given as late as 6 hrs after bretylium, the latency between the injection of DMI and the T_{50a} of the group was short In the 11—15 hrs group the latency was longer and in the 11—13 hrs group the latency (here perhaps not quite comparable) was even longer Thus, the later DMI was given the faster the degeneration contraction appeared after the injection of DMI

If the time interval between the injections of bretylium and DMI was fixed to 2 hrs but the times of injections in relation to denervation were varied, see Fig 5

TABLE VI Influence of varying injection times of bretylium and Lu 3-010 on the delay reducing effect of Lu 3-010 The drugs were given subcutaneously, bretylium (4 mg kg) always before Lu 3-010 (10 mg kg) Abbreviations used in the squares T=time of start of degeneration contraction ($T_{50\%}$), $T_{50\%}$ is the time of half maximum development of the degeneration contraction D=delay of degeneration contraction calculated as the difference between the mean $T_{50\%}$ *of the experimental group and that of the group of untreated* rats L=latency between the injection of Lu 3-010 and the degeneration contraction calculated as the difference between the mean $T_{50\%}$ of the group and the mean time of Lu 3-010 injection The values are M±S E M expressed in hours There were 5 rats in each group, if not otherwise stated For analysis of significance Student's t test was used In the column 'groups compared' the time of bretylium injection is mentioned first thus 11—13 means bretylium at 11 and Lu 3 010 at 13 hrs after denervation

Time of Lu 3-010 injection[1]	Time of bretylium injection[1]			Reliability of difference between means p values			
	11	13	No bretylium =NB	*Groups compared*	$T_{50\%}$	*Delay*	*Latency*
	T 15.82±0.17[1]			11—13 with 13—15	<0.01	<0.01	<0.01
13	D 0.65±0.17			11—13 with 11—15	<0.001	<0.001	<0.001
	L 2.82±0.17			11—13 with 11—NL	<0.001		
				11—13 with NB—NL	n.s.		
	T 16.91±0.11	T 16.99±0.20		11—15 with 13—15	n.s.	n.s.	n.s.
15	D 1.74±0.11	D 1.82±0.20		11—15 with 11—NL	<0.001		
	L 1.80±0.08	L 2.09±0.14 n=10		11—15 with NB—NL	<0.001		
				13—15 with 13—NL	<0.001		
No Lu 3-010 =NL	T 20.31±0.25	T 20.51±0.33	T 15.17±0.11	13—15 with NB—NL	<0.001		
	D 5.14±0.25 n=10	D 5.34±0.33 n=10	n=49				

[1] time of injection and time of start are expressed in hours after denervation

and Table V, the latencies of the groups did not differ significantly The delays of the start of the degeneration contraction consequently were greater the later the drugs were given

In the 2 groups where the times of bretylium injection differed (11 or 13 hrs) but those of DMI injection were fixed at 15 hrs the latencies also differed, see Table Fig 4 and 5 In the 11—15 hrs group which had the longest interval betw injections, the latency was significantly shorter than that of the 13—15 h

TABLE V Influence of varying injection times of bretylium and DMI on the delay reducing effect of DMI The drugs were given subcutaneously, bretylium (4 mg/kg) always before DMI (10 mg/kg) Abbreviations used in the squares T=time of start of degeneration contraction ($T_{50\%}$), $T_{50\%}$ is the time of half maximum development of the degeneration contraction D=delay of degeneration contraction calculated as the difference between the mean $T_{50\%}$ of the experimental group and that of the group of untreated rats L=latency between the injection of DMI and the degeneration contraction calculated as the difference between the mean $T_{50\%}$ of the group and the mean time of DMI injection The values are M ± S E M expressed in hours There were 5 rats in each group if not otherwise stated For analysis of significance Student's t test was used. In the column groups compared, time of bretylium injection is mentioned first thus 10—12 means bretylium at 10 and DMI at 12 hrs after denervation

Time of DMI injection in hours[1]	Time of bretylium injection in hours[1]: 10	11	13	No bretylium =NB	Reliability of difference between means p values: Groups compared	$T_{50\%}$	Delay	Latency
	T 15.74				10—12 with	n s	n s	n s
	±0.26				11—13			
12	D 0.57				10—12 with	<0.001		
	±0.26				10—ND			
	L 3.72				10—12 with	n s		
	±0.27				NB—ND			
		T 16.43			11—13 with	<0.001	<0.001	n s
		±0.28			13—15			
13		D 1.26			11—13 with	<0.05	<0.05	<0.05
		±0.28			11—15			
		L 3.43			11—13 with	<0.001		
		±0.27			11—ND			
					11—13 with	<0.01		
					NB—ND			
		T 17.34	T 18.35		11—15 with	<0.05	<0.05	<0.05
		±0.19	±0.24		13—15			
15		D 2.17	D 3.18		11—15 with	<0.01	<0.01	<0.001
		±0.19	±0.24		11—17			
		L 2.35	L 3.28		11—15 with	<0.001		
		±0.19	±0.25		11—ND			
			n 10		11—15 with	<0.001		
					NB—ND			
		T 18.22			13—15 with	<0.01		
		±0.07			13—ND			
17		D 3.05			13—15 with	<0.001		
		±0.07			NB—ND			
		L 1.19			11—17 with	<0.001		
		±0.08			11—ND			
					11—17 with	<0.001		
No DMI	T 18.98	T 20.31	T 20.51	T 15.17	NB—ND			
=ND	±0.27	±0.25	±0.33	±0.11				
	D 3.81	D 5.14	D 5.34					
	±0.41	±0.25	±0.33					
	n=10	n=10	n=10	n=49				

[1] time of injection and time of start are expressed in hours after denervation

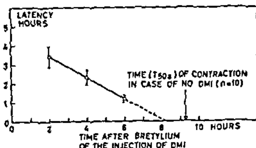


Fig 6 Effect of varying time intervals between the injections of bretylium and DMI on the time needed for DMI to start the degeneration contraction (the latency) The drugs were given subcutaneous h bretylium (4 mg/kg) always at 11 hrs after denervation and DMI (10 mg/kg) 2 4 or 6 hrs later $T_{50\%}$ is the time of half maximum development of the degeneration contraction The latency is calculated as the difference in hours between the mean $T_{50\%}$ of the group and the mean time of DMI injection Each point represents the mean of 5 rats The vertical bars show S E M The linear extrapolation onto the abscissa is shown by the dotted line.

Table VI Lu 3 010 tended to have a stronger delay-reducing effect than DMI The main pattern of effects was similar to that of the DMI-experiment Thus, the latency was shorter the longer interval between the injection of bretylium and Lu 3 010, compare the 11—15 and 11—13 hrs groups, the delay was longer the later the drugs were injected, compare the 11—13 and 13—15 hrs groups But there were differences too The degeneration contraction of the 13—15 hrs group started comparatively early, i e 1 36 ± 0 22 hrs earlier than that of the 13—15 hrs group in the DMI experiment The latency of the 13—15 hrs Lu group was 0 73 ± 0 22 hrs shorter than that of the 11—13 hrs Lu group while with DMI there was no significant difference between the corresponding groups The significance of this possible difference in action between the two drugs is at present hard to evaluate

These findings with DMI and Lu 3-010 seem to indicate that bretylium looses its delaying action when a sufficient low level at the critical sites is reached by continuous disappearance and that DMI or Lu 3 010 subsequently given in some way or another, increases the rate of the bretylium loss Another explanation would be that the start of the delayed degeneration contraction is due to a catching up by a bretylium resistent mechanism of degeneration release of transmitter which is accelerated by DMI or Lu 3-010

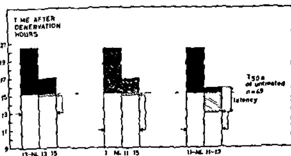


Fig 7 Influence of varying injection times of bretylium and Lu 3 010 on the delay reducing effects of Lu 3 010 The drugs were given subcutaneously bretylium at 4 mg/kg and Lu 3 010 at 10 mg/kg The filled and open arrows show the injection times of bretylium and Lu 3 010 respectively The bars represent the mean $T_{50\%}$ of the group which is the time of half maximum development of the degeneration contraction The black part of the bars is the bretylium induced delay The designations of the groups placed below the bars in the figure are the same as th used in Table VI NL means no Lu 3 010 The groups marked 11 15 and 11 13 had n the other groups had n = 10

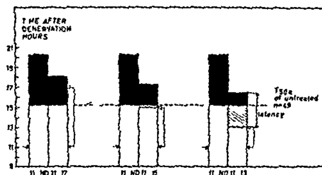


Fig 4 Delay reducing effect of DMI injected at varying time intervals after bretylium given at 11 hrs after denervation The drugs were given subcutaneously bretylium at 4 mg/kg and DMI at 10 mg/kg The filled and open arrows show the injection times of bretylium and DMI respectively The bars represent the mean T_{50_2} of the group which is the time of half maximum development of the degeneration contraction The black part of the bars is the bretylium induced delay The designations of the groups, placed below the bars in the figure, are the same as those used in Table V, ND means no DMI The group marked 11 ND had n=10 the other groups had n=5

Thus, with the drugs given within 10 to 15 hrs after denervation the latency between the injection of DMI and the T_{50_2} appears to be related to the time interval between the injections of bretylium and DMI but not to the intervals between the denervation and the injections In Fig 6 the latencies of the 11—13 11—15 and 11—17 hrs groups are plotted against the corresponding intervals between the injections of bretylium and DMI It is seen that a linear extrapolation (dotted line) intersects the abscissa at about 8 5 hrs, which is strikingly close to the start of the phenomenon when 4 mg/kg bretylium is given alone at 11 hrs, 9 31 ± 0 2) (mean and s e m)

Experiments with Lu 3 010

In similar experiments Lu 3 010 was studied instead of DMI Lu 3 010 is known to be a potent and comparatively specific inhibitor of the axonal amine pump of adrenergic nerves lacking for instance any anticholinergic action (Petersen et al 1966 Waldeck 1968 Carlsson et al 1969) The results are shown in Fig 7 and

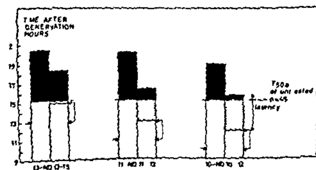


Fig 5 Delay reducing effect of DMI always injected 2 hrs after bretylium given at varying time intervals after denervation The drugs were given subcutaneously bretylium at 4 mg/kg and DMI at 10 mg/kg The filled and open arrows show the injection times of bretylium and DMI respectively The bars represent the mean T_{50_2} of the group which is the time of half maximum development of the degeneration contraction The black part of the bars is the bretylium induced delay The designations of the groups placed below the bars in the figure are the same as those used in Table V ND means no DMI The groups marked 11—13 and 10—12 had n=5 the other groups had n=10

Discussion

Inhibition of the axonal amine pump mechanism and the start of the degeneration contraction

Hitherto only a few authors have paid attention to the processes initiated by axotomy of the sympathetic nerves and responsible for the degeneration transmitter release Malmfors and Sachs (1965) however proposed from their histochemical study that the initial and greatest part of the postdenervation loss of transmitter from the nerve endings in the rat iris was due to sudden degenerative changes in the axon membrane Simultaneously the amine uptake mechanism at this level seemed to deteriorate but this was suggested not to be the sole cause of the start of the transmitter release In the present investigation it was shown that pre treatment with desmethylimipramine (DMI) alone which is a potent inhibitor of the axonal amine pump mechanism (Malmfors 1965 Carlsson and Waldeck 1965a) did not start the degeneration contraction prematurely This indicates that an isolated dysfunction of the membrane pump cannot alone be responsible for the initiation of the degeneration contraction Lu 3 010 started the contraction somewhat prematurely but the results with DMI at increasing doses indicate that this is due to some extra property of Lu 3 010 rather than a more complete block of the membrane pump

The inhibiting effect of imipramine like agents on the bretylium induced delay

The blocking effect of bretylium and related agents on the release of the transmitter by nerve impulses or different drugs are known to be antagonized by several substances such as cocaine (Nasmyth and Andrews 1959 Boura and Green 1959) certain indirectly acting sympathomimetic amines (Wilson and Long 1960 Laurence and Rosenheim 1960 Matsumoto and Horita 1962 Day 1962 Chang 1965 Chang *et al* 1965) and imipramine (Gokhale *et al* 1965 and Gokhale *et al* 1966) The mechanism of the antagonism to nerve blockade by these drugs is still almost unknown It was found in the present study that drugs with imipramine like activity *i e* DMI protriptyline and Lu 3 010 which is a bicyclic phthalan derivative were found promptly to interact with bretylium, *i.e.* to reduce the bretylium induced delay Lu 3 010 tended to be the most potent of the drugs DMI which also was given before bretylium almost completely prevented the development of the delaying action of bretylium Trimipramine or cocaine neither reduced the bretylium induced delay nor prevented its development Dexamphetamine and metaraminol also have the ability to reduce the delaying effect of bretylium (Lundberg 1969b) Cocaine DMI protriptyline and Lu 3-010 are well known to be effective inhibitors of the amine uptake mechanism at the level of the sympathetic axon membrane (Furchgott *et al* 1963 Hillarp and Malmfors 1964 Malmfors 1965 Carlsson and Waldeck 1965a Waldeck 1968 Carlsson *et al* 1969 The activity of cocaine in vivo is distinctly weaker than those of the three other drugs which are considered to be almost equally potent at 10 mg/kg (Carlsson and Waldeck Waldeck 1968) Trimipramine although structurally closely related to DMI

pletely lacks such an effect (Bickel and Brodie 1964, Carlsson and Waldeck 1965b). The observations in the present experiments that DMI, protriptyline and Lu 3-010 clearly reduced the bretylium-induced delay and that trimipramine had no effect point to an inhibition of the membrane pump as being involved in the antagonism. But the finding that cocaine prolonged the delaying effect of bretylium instead of antagonizing it seems at first sight not to accord with such a postulate. Recently, however, results have been published which indicate that the action of cocaine is more complex in nature than earlier considered and which necessitate more cautious interpretation of results from experiments with cocaine. For example, Peterson et al (1966) who performed a comparative in vivo study of several drugs with imipramine-like activity in the rat and mouse, found that cocaine had much weaker reserpine antagonizing activity than Lu 3-010, protriptyline and DMI but potentiated exogenous noradrenaline comparably well. Moreover, Kalsner and Nickerson (1969) from experiments with their oil immersion technique proposed that the major part of the potentiation of noradrenaline by cocaine is due to an effect on the effector cell rather than to an action related to inactivation of the amine, that is to the function of the membrane pump mechanism. Friedman and Bhagat (1968) found differences between the actions of subcutaneously injected DMI and cocaine in the rat. DMI at 2.5 mg/kg decreased the accumulation of tritiated noradrenaline in the rat heart during increased sympathetic activity associated with cold, whereas cocaine at 10 mg/kg was ineffective.

The hypothesis that the site of interaction between bretylium and DMI like drugs studied in the present investigation is located to the cell membrane has to contend with other difficulties. First, although being strongly ionized at physiological pH values and virtually lipid insoluble, bretylium has been shown to accumulate in the rat heart by a mechanism which is hard to saturate and not blockable by DMI. This indicates that bretylium does not need to use the axonal amine pump for its entrance into the adrenergic nerve (Brodie et al 1965). Second, Brodie et al (1968) proposed that DMI prevents the amphetamine-induced release of noradrenaline through an action within the adrenergic neuron since the liposoluble amphetamine enters the nerve cell by simple diffusion (Ross and Renyi 1966). These findings thus leave the possibility open that the bretylium antagonizing effect of DMI reported above is excerted intraneuronally or at sites in the axon membrane different from those related to the amine pump mechanism. In a recent report Reid et al (1969) have presented further evidence for an intraneuronal action of DMI.

It is known that labelled bretylium is selectively accumulated in sympathetic nerves and retained there at high concentrations for a long time, in the cat for more than 18 hrs (Boura et al 1960), and that it is almost not metabolized in the tissues (Duncombe and McCoubrey 1960). Since bretylium causes a considerable delay when injected as late as 13 hrs after ganglionectomy it is reasonable to assume that the drug is concentrated to the sympathetic neuron even at that time. It is also plausible that bretylium then spontaneously or as a consequence of proceeding degeneration leaves the critical sites in the axons by and by. When the degeneration contraction starts

this can be due to either a sufficiently low level of bretylium or to the fact that another process that also causes degeneration release of transmitter and is not blockable by bretylium, takes over As is shown above, a second injection of bretylium given at 14 or 17 hrs after denervation significantly prolongs the delay caused by a single injection given at 10 or 13 hrs respectively Thus with bretylium injected within 10 to 17 hrs after denervation which is the period studied in the present investigation it seems probable that the onset of a delayed degeneration contraction is due to disappearance of bretylium rather than to catching up by a bretylium resistent mechanism of the transmitter release This hypothesis is also favoured by the results of the present experiments in which the consequences of varied times if injections of bretylium and DMI on the delay reducing effect of DMI were studied the latencies between the injection of DMI and the start of the degeneration contraction were then seen to differ The latency did not seem to be correlated to the intervals between the denervation and the injections of the drugs but to the interval between the injections of bretylium and DMI The latency declined with increasing intervals between the two injections The tempting explanation for this effect is that bretylium after being taken up into the nerve endings then disappears from the critical sites and the longer time elapsed after the injection of bretylium the lesser amount of bretylium is left in the nerve terminals The way of action of DMI in this situation may be to increase in some way or another the rate of bretylium loss from the critical sites Since it is suggested above that DMI reduces the bretylium induced delay by a mechanism which is non competitive in nature DMI does not seem to induce an increased rate of bretylium loss by competing for the receptor sites mediating the delaying action of bretylium that is simple displacement but by an action at other hypothetical sites, maybe thereby inhibiting the re uptake of bretylium

It is known that DMI and Lu 3 010 have somewhat different actions Lu 3 010 is considered to be of about the same potency as DMI but a more specific inhibitor of the membrane pump lacking for instance almost any anticholinergic activity which characterizes the tricyclic anti depressant drugs (Petersen *et al* 1966 Waldeck 1968) Also in the present study the effects of Lu 3 010 and DMI differed to some extent First Lu 3 010 in general tended to be more potent than DMI in reducing the bretylium induced delay Second the delay induced by bretylium given 2 hrs before Lu 3-010 was more efficiently reduced by Lu 3 010 injected at 15 hrs than at 13 hrs after denervation while with DMI the time interval between the denervation and the injection did not significantly influence the delay reducing action Third when given alone Lu 3 010 caused the degeneration contraction to start somewhat prematurely as distinguished from DMI which did not influence the onset of the contraction even at a dose twice that of Lu 3 010 These findings point out that Lu 3 010 compared to DMI has some additional action on the degenerating adrenergic nerve terminals However the nature of this action is at present hard to evaluate

Conclusions

The onset of the degeneration contraction does not seem to be initiated only by dysfunction of the amine pump mechanism at the level of the adrenergic axon membrane

Drugs known to inhibit the axonal membrane pump, i e DMI, protriptyline an[illegible] Lu 3-010 but not cocaine, effectively antagonize the delaying action of bretyl[illegible] However, at present it does not seem possible to explain the antagonism to bretyl[illegible] by simple interference with the amine pump of the adrenergic axon membrane.

The start of the degeneration contraction delayed by bretylium given before th[illegible] expected start of the contraction is probably due to a sufficiently low level of th[illegible] drug at the critical sites rather than to a catching-up by a bretylium resist[illegible] mechanism of degeneration release of transmitter

DMI and Lu 3-010 seem to have somewhat different actions on the degenera[illegible] adrenergic nerve endings.

I am most grateful to Professor E Bárány for instructive and stimulating discussions and [illegible] Mrs L Bennich Bjorkman and Mrs J Wersall for skilful technical assistance
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Histochemical and Biochemical Correlations of Monoamine Oxidase Activity in Autonomic and Sensory Ganglia of the Cat

By

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Abstract

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The presence of monoamine oxidase (MAO) in a wide variety of tissue although rules out its exclusive association with adrenergic neurons Nevertheless considering its role in the regulation of the catecholamine (CA) content of the adrenergic neurons one may suspect that a high MAO activity is characteristic for these cells Koelle and Valk (1954) however when comparing histochemically the MAO activity of different ganglia did not find any correlation between MAO activity and adrenergic function in the peripheral nervous system of the cat The successive development of highly sensitive and specific methods for the measurement and localization of CA as well as of MAO activity (McCaman *et al* 1965, Consolo *et al* 1968) encouraged us to reinvestigate this problem

Material and methods

Adult cats of both sexes were anesthetized with ether or with sodium pentobarbitone (Nembutal Abbott, 40 mg/kg i p) For the biochemical assay the excised ganglia were rapidly freed from their capsules weighed and homogenized in a glass homogenizer MAO activity was

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TABLE I MAO activity and catecholamine content of autonomic and sensory ganglia of the cat

Type of ganglion	MAO activity[1]	Catecholamine content	References
		Fluorescent histochemical results	
Superior cervical	6 64 ± 0 62 (5)	Great majority of neurones exhibit fluorescence	Norberg and Hamberger 1964
Stellate[2]	6 6 ± 0 8 (6)		Hamberger *et al* 1965 a
L_7[2]	4 2 ± 0 3 (13)		Giacobini *et al* 1969
Nodose	3 51 ± 0 28 (6)		Nielsen *et al* 1969
Spinal dorsal root ganglion	1 47 ± 0 08[3] (6)	Neurones do not display any fluorescence	Owman and Santini 1966
Ciliary	2 05 ± 0 18[3] (6)	Only few fluorescent terminals are visible	Hamberger *et al* 1965 b

[1] MAO activity expressed in μmoles of product/hr g/wet weight The values represent M ± S E M Number of cats within parenthesis

[2] Consolo *et al* Acta physiol scand 1968 *74* 513—520

[3] These values are significantly lower than that of L_7 ($p < 0\ 001$)

measured according to Consolo *et al* (1968) using ^{14}C tyramine For the histochemical localization of MAO activity 5 10 and 20 μ thick cryostat sections were incubated for two hrs according to Glenner *et al* (1957)

Statistical treatment of the results

The MAO activity of the different ganglia were compared with that of L_7, L_7 was used for comparison since the percentage of cholinergic and adrenergic cells has been biochemically established (Giacobini *et al* 1967 Buckley *et al* 1967 Consolo *et al* 1968 Giacobini *et al* 1969) The significance of the differences was calculated according to the t test of Student

Results

Biochemical results

The values of MAO activity for the different autonomic and sensory ganglia are given in Table I The sympathetic ganglia exhibited the highest activity The MAO activity values for the parasympathetic ciliary and the sensory dorsal root ganglia were significantly lower ($p < 0\ 001$) than that of the L_7 No difference was observed between spinal ganglia originating from various levels of the spinal cord The activity of the nodose ganglion was only moderately below the value reported for L_7

Histochemical observations

Sympathetic ganglia (Fig 1 and 2) the same staining pattern characterized superior cervical stellate and L_7 ganglia The cytoplasm of the cells exhibited a uniform lila colouring The neuronal perikarya were distinguished by a large number of

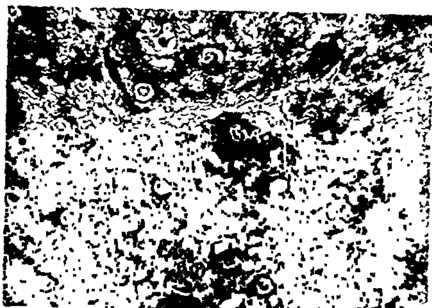


Fig. 1. MAO activity in the superior cervical ganglion, cat. The cytoplasm of both nerve (N) and glial (G) cells exhibit a diffuse lilac colouring. The neurones are distinguished by a large number of dark blue granules scattered *in their cytoplasm.* ×420

stained dot-like granules scattered in the cytoplasm. The reaction intensity in the individual nerve cells varied considerably. Glial cells displayed only moderate activity.

Nodose ganglion (Fig. 3): the colour of the cytoplasm was weaker, the number of granules was less than in the sympathetic ganglia. The satellite cells exhibited the same staining intensity.

Spinal dorsal root ganglia: the staining pattern was similar to that of the nodose ganglion though the intensity of the reaction was much weaker. No granular reaction could be detected in the nerve cells.



Fig. 2. MAO activity in the superior cervical ganglion, cat. With higher magnification the dot-like appearance of the granules reflecting mitochondrial enzyme activity is clearly visible. ×630

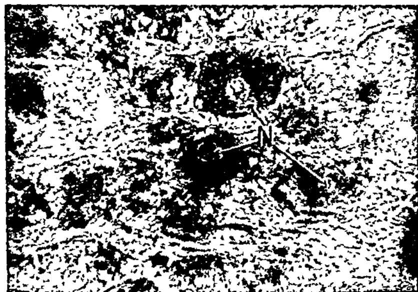


Fig 3 MAO activity in the nodose ganglion cat The cytoplasmic reaction is weaker and the number of granules within the neurons (N) is less than in the superior cervical ganglion ×420

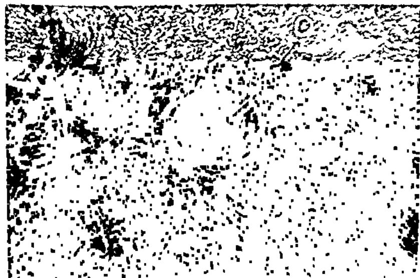


Fig 4 MAO activity in the ciliary ganglion, cat The neurons (N) display only faint cytoplasmic reaction The granules are completely absent from the cells The marked colouring of the myelinated axons numerous only in the ciliary ganglion, is a staining artifact due to strong lipid solubility of the reaction product ×420

Ciliary ganglion (Fig 4) all the cells displayed a very faint lilac colouring the blue dot like reaction was completely absent from the perikarya The staining of the numerous myelinated axons in the neuropile is considered to be an artifact reflecting strong lipid solubility of the reaction product

Discussion

Using the fluorescence histochemical technique the distribution of adrenergic neurons in the peripheral nervous system has been accurately mapped Para sympathetic and sensory neurons do not display fluorescence (Norberg and Hamberger 1964, Hamberger *et al* 1965a) In the sympathetic ganglia two populations of neurons can be distinguished The majority of cells show a strong fluorescence and weak acetylcholinesterase (AChE) activity, the minority is devoid of CA and exhibit an intense AChE reaction (Hamberger *et al* 1965a Giacobini *et al* 1967 Giacobini *et al* 1969) These latter cells were postulated by Sjoqvist (1962) to be of cholinergic nature

The varying percentage of adrenergic neurons within the sympathetic ganglia could be correlated with their CA content (Norberg and Sjoqvist 1966) The CA content in turn correlates well to the MAO activity of the different ganglia (Consolo *et al* 1968) This relationship is in agreement with the very low MAO activity in the parasympathetic and sensory ganglia found in the present investigation The relationship is however not so striking in the case of the nodose ganglion These cells do not display any specific fluorescence (Norberg and Hamberger 1964 Hamberger *et al* 1965a Nielsen *et al* 1969), but show a measurable though considerably lower MAO activity than the sympathetic ganglia This difference in MAO activity becomes especially evident comparing the nodose with the superior cervical ganglion According to Sjoqvist (1962) the cholinergic neurons are not so numerous in the latter ganglion

The histochemical localization of MAO activity firmly supports these measurements A great variation of the frequency of the dot like blue granules within the different cell types represents the most significant feature of our pictures These granules correspond most probably to formazan precipitation along the mitochondrial membranes (Ogawa and Barrnett 1964—1965, Seligman *et al* 1961)[1]

The faint cytoplasmic reaction which is completely absent in substrate-free controls is caused by a slight diffusion of the reaction product Considerably more precipitate is necessary to produce sufficiently dark colour for the safe detection of the small dot like reaction than for the visualization of a diffuse cytoplasmic colouring

[1] After this paper was accepted for publication a paper appeared about the histochemical localization of MAO activity in electron microscopy using thiocarbamyl nitro blue tetrazolium (Boadle and Bloom 1969 This paper confirms our view that the granules correspond to formazan precipitations along the outer mitochondrial membrane No other areas showed any sign of reaction therefore the faint cytoplasmic staining can be attributed to diffusion artefacts

Therefore the frequency of the reacted mitochondria is the only safe guide to assess the MAO content of a cell

The adrenergic neurons in our pictures are filled with reacted mitochondria causing their intense blue colouring which sharply contrasts with the pale lilac reaction of the glial cells The granules are completely absent in the ciliary and spinal ganglia but a very weak cytoplasmic reaction could indicate a low activity of these cells in agreement with the biochemical data

Within the sympathetic ganglia considerable activity differences between individual neurons could be detected Similar variation of the fluorescence intensity from one cell to another was observed by Norberg and Hamberger (1964) and Hamberger *et al* (1965a) Perikarya completely devoid of active mitochondria as seen in the ciliary ganglion were not encountered The histochemical studies of the above authors and the determination of MAO activity and CA fluorescence of dissected single cells from the L_7 ganglion (Consolo *et al* 1968, Giacobini *et al* 1969) indicate that approximately 10—15 per cent of the total cell population lacks CA and measurable MAO activity

It is interesting to compare our histochemical results with those of Koelle and Valk (1954) since the techniques applied are completely different The method of these authors is based on the simultaneous coupling of the aldehyde produced during deamination, whereas in that of Glenner *et al* (1957) the tetrazolium salt Nitro BT is reduced directly or indirectly following deamination to yield blue formazan precipitate Both methods were proved by means of different inhibitors to be specific for MAO In both cases a diffuse cytoplasmic staining is present Kolle and Valk (1954) based their conclusion on this cytoplasmic colouring paying little attention to the scattered pigment granules frequently and exclusively encountered in the neurons of the sympathetic ganglia Most probably their significance was not appreciated because of lack of evidence at that time that these granules correspond to mitochondrial enzyme activity If the frequency of the granules is evaluated assuming that these granules correspond to the mitochondrial enzyme reaction there is no difference between the results of the two histochemical studies

The data presented here indicate that the majority (80—90 per cent) of neurons in the sympathetic ganglia display high MAO activity MAO activity on the other hand seems to be specifically associated with the high CA content present in the adrenergic neurones (Giacobini *et al* 1969 It is hazardous to extrapolate these results to the central nervous system Nevertheless the strong histochemically demonstrable MAO activity in the locus coeruleus (Shimizu *et al* 1958) and median eminence (Matsui and Kobayashi 1965) where numerous fluorescent perikarya (Dahlstrom and Fuxe 1964) and axon terminals respectively (Fuxe 1964) are present allows to suggest that this correlation might hold for the central nervous system as well

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Rate Changes in Spontaneous Neuronal Impulse Sequences

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Abstract

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Sudden rate changes in spontaneous activity were seen in 16 cells of 373 examined under light urethane anesthesia in various diencephalic, striatal, and limbic areas of rabbits. The stochastic properties of the rate varying impulse sequences were examined with statistical techniques. In this material the dispersion of impulse interval lenghts was a uniform function of the mean discharge rate. For a range of spontaneous activity from 1.5 to 73 imp/sec this relation was adequately expressed by the equation $s = 1.42 \cdot m^{1.42}$, where m is the mean interval in seconds and s is the standard deviation of the interval lengths in seconds. This relation held for means and standard deviations from different cells as well as for different values from the same cell. In one half of the cells, a time series analysis revealed no change in the stochastic properties of the impulse sequence at the time of the rate change. In other cells, a periodic rhythmic activity was disclosed during slower discharge rates but it was not present in the faster activity, or vice versa. In yet other cells, the changes in mean discharge rate were paralleled by similar changes in the frequency of the rhythm. Except for the rhythmic periodicity all the stochastic properties examined were related to the mean frequency of firing.

The most commonly held view of the spontaneous activity of nerve cells is that it acts as invariant background noise on which the signals of stimuli are superimposed (for a review see Granit 1955). In the visual system, however, variations in the level of the spontaneous activity in darkness have been described in the retinal ganglion cells (Rodieck and Smith 1966), in lateral geniculate cells (Lewick and Williams 1964) and in cortical cells (Griffith and Horn 1966). When we observed sudden changes in the rate of the spontaneous activity in cells of various diencephalic, striatal and limbic areas of anesthetized rabbits we wondered about the significance of these changes. Obviously a faster rate in such a cell could represent activation of an excitatory cell in a circuit involved in automatic control of body functions or it could represent a change in the excitability of the region of recording, the activity remaining spontaneous in nature. By studying the stochastic properties of the impulse sequences of different discharge rates we tried to determine whether the faster

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activity in each cell represented a specific driving or whether the nature of the a tivity remained spontaneous also in the faster rates Basic to this approach was th description by Werner and Mountcastle (1963) of the differences in the variabili impulse intervals between the spontaneous and driven activities and the demons tion by Poggio and Viernstein (1964) of variation of the time structure of impul sequences with various degrees of driving In order to see whether such differenc could be detected between the slower and faster spontaneous impulse trains fro each neuron, we analyzed the dispersion of impulse intervals and the tempor structure of the discharge In this material, the stochastic properties of rate varyi spontaneous impulse trains did not fall into two categories that could be described spontaneous and driven activity, but they formed a smooth continuum as a functio of mean discharge rate to which the changes in the time structure also seemed to l linked

Methods

The material for this study was collected among recordings from 373 cells studied for p previous papers (Hyvarinen 1966 Hyvarinen Heino and Haikara 1967) Activity of the cells was recorded extracellularly with glass capillary microelectrodes in various diencepha striatal, and limbic areas of the rabbit brain under light urethane anesthesia. Parietal elec ... ordings the laboratory ... d at 37±1° C ... ded in these stud ... s to observe that th ... ording varied from ... aluation of the t ... to 60 min per cell with longer periods for slower activity ... changes all impulse sequences were photographed from FM tape as dot displays of the i pulse intervals Selected sequences were then digitized with a resolution of 1 msec and sta cal analyses carried out with a μ LINC computer

In addition to the examination of the dispersion of interval lengths as a function of me interval we studied the stochastic properties of the time structure of the impulse sequences constructing interval histograms and computing conditional probability densities of impul occurrence after any impulse The latter function has been called expectation density (Poggi and Viernstein 1964) and intensity function (Cox 1965) the latter name is used in th report The intensity function is closely related to the autocorrelation function and can ... represents the stimulus The ... studied impulse sequenc ... process the order of t ... ty function For a renew ... e periodically osci ... order of intervals prev ... process the intensity fu ... he sequence The inten ... d compared with the r ... ndom shuffle of the se ... and the renewal dens ... dependent process For reflect the existence of a specific serial order of intervals a time dependent process For renewal process the two functions follow an identical time course For a Poisson process t probability of impulse occurrence is constant in time and hence the intensity function and t renewal density coincide in a horizontal line

We computed interval histograms renewal densities and intensity functions of the t different states of activity for each cell We used the integral equation of renewal theory (C ... from the interval histograms On the figures the ... are plotted as bar graphs and intensity functions

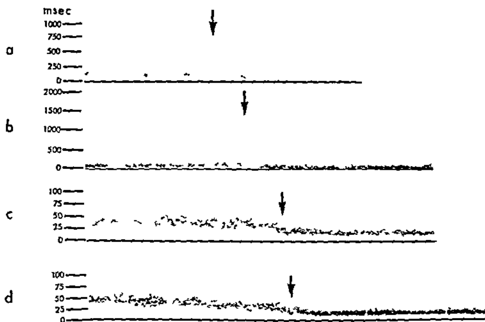


Fig 1 Digital dot plots of impulse intervals about the changes in mean discharge rates (arrows) The height of a dot from the baseline indicates the length of an interval Stationary sequences on opposite sides of the arrows were selected for statistical analysis *a* Cell 92302 recorded in the hippocampus of an adult rabbit (Other results for this cell are shown in Fig 3) *b* Cell 81102 recorded in the internal capsule of a 1 month old rabbit (See Fig 4) *c* Cell 47101 recorded in the thalamic nucleus reticularis of a 2 month old rabbit See Fig 5) *d* Cell 44402 recorded in the globus pallidus of a 3 month old rabbit (See Fig 6)

Results

Among the spontaneous impulse sequences from 373 neurons 16 units were found (4 %) which displayed sudden accelerations or decelerations during the limited recording periods These cells were found in 13 rabbits ranging in age from 18 days to 8 months and these cells were randomly distributed in the various brain regions mentioned in the original studies

of the rate change no signs of change were detected in the monitored waveform or in the audible pitch of the spike Slight movements of the electrode did not influence these changes The animals were sleeping unperturbed under the anesthetic and no stimuli were applied No clear changes were observable in the electrocorticogram at the times of the rate changes In the samples of activity of these cells the mean discharge rates ranged from 1 impulse per 20 sec to 73 imp/sec with a mean of 16 imp/sec The observed rate changes ranged from 0 2 to 300 times the slower mean rate recorded in each cell Only two clearly different levels of activity were observed in any cell during the limited observation periods

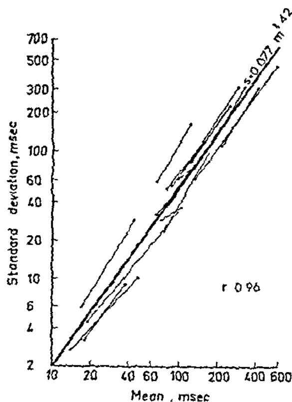


Fig 2 Standard deviation of i[…] interval lengths as a function of […] interval in double logarithmic […] second scales The heavy line […] the best fitting regression line […] two values from the two different […] of activity in each cell are join[…] thin lines Correlation coefficien[…] 0 96

As the dot displays indicated stationary activity in all samples and as the int[…] histograms (Fig 3 5 and 6) were sufficiently regular for 14 cells standard devia[…] of impulse intervals were selected as indicators of the variability of the activit[…] two cells bimodal histograms were observed (Fig 4) they were not included i[…] analysis of interval dispersion The means (m) and standard deviations (s) of […] pulse intervals were computed for the 14 cells in the two observed states They […] plotted in Fig 2 on double logarithmic co-ordinates The logarithmic transfor[…] the data indicates a linear relation of the log s to log m The correlation coeffic[…] for the logarithmic values is 0 96 When the mean and standard deviation are […] pressed in seconds the equation for the best fitting regression line is s = 1 42 m […] That the two constants in this geometric relationship happen to be identical u[…] two decimals is a conspicuous coincidence If the data are expressed in millisecο[…] the value of the multiplier of m is reduced to 0 0765 The residual standard devia[…] of s in the logarithmic form is 0 15

In Fig 2 the heavy line shows the best fitting regression line to all the points […] thin lines join the two pairs of values from each cell measured before and after […] rate change The standard deviation follows closely the above relationship also w[…] the activity changes from one level to another in any one cell

The non linearity of this relationship is seen in the coefficients of variation (i/[…]

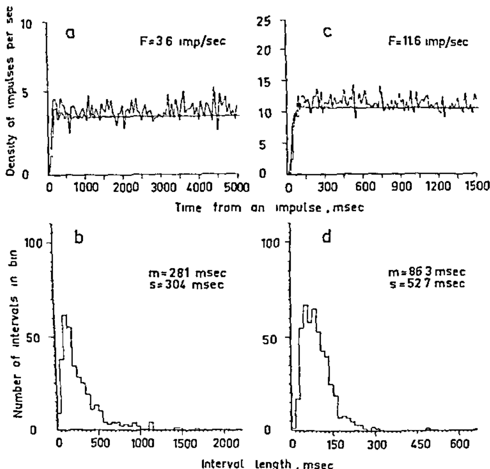


Fig 3 An example of the constancy of time structure in spite of change in mean frequency of firing (F) Figs a and b before rate change and c and d after it Cell 92302 (Fig 1 a) changes its firing rate from 3 6 (*a* and *b*) to 11 6 imp/sec (*c* and *d*) but the form of the interval histogram (*b* and *d*) as well as the intensity function (*a* and *c*) remains unchanged and indicates a renewal process which is Poissonian although very short intervals are absent

which for fast activity corresponding to the lower left corner of Fig 2 have values around 0 20 and which for slower activity change gradually to values around 1 30 For the coefficient of variation (CV) the following equation would hold CV = 1 42 m [.42] when *m* is expressed in seconds

Fig 3—6 illustrate the changes observed in the interval histograms intensity functions and renewal densities at times of the rate changes The most common observation illustrated in Fig 3 was that there was no change in the stochastic properties of the impulse sequence when the rate change took place In Fig 3 the forms of the interval histograms and the intensity functions are quite similar before and after the mean rate change which was from 4 to 12 imp/sec No change was observed in 8 of

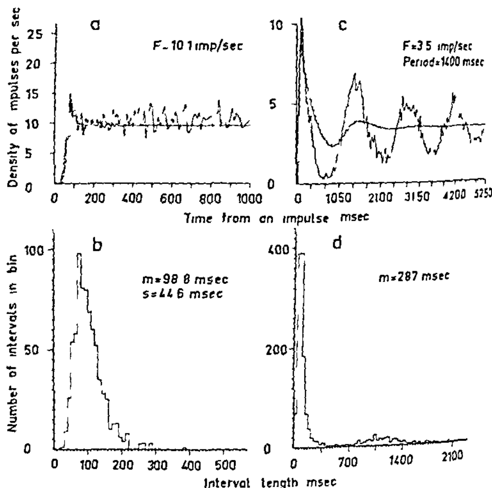


Fig 4 An example of a change from a rapid renewal process to a slower time dependent periodic process in cell 81102 (Fig 1 b) When the mean rate falls from 10 1 to 3.5 imp/sec the interval histogram becomes bimodal and the intensity function distinguishes itself from the renewal density

the 16 cells studied In all these cells the process was a renewal proces on both levels of activity as illustrated by the fact that the intensity function and the renewal density followed similar time courses

In 4 cells a rhythmic periodicity was observed in the slower discharge rate In the faster rate the periodicity disappeared and the process became a renewal process (Fig 4) In one cell a change in the opposite direction was seen In 3 cells the activity was periodic both before and after the rate change In these cells parallel with the change in the mean frequency of firing a corresponding change happened in the frequency of the probability oscillations In these sequences the mean frequency was always the same as the frequency of the probability oscillations Fig 5 shows an example of such a change in a cell in which the activity was a renewal process both

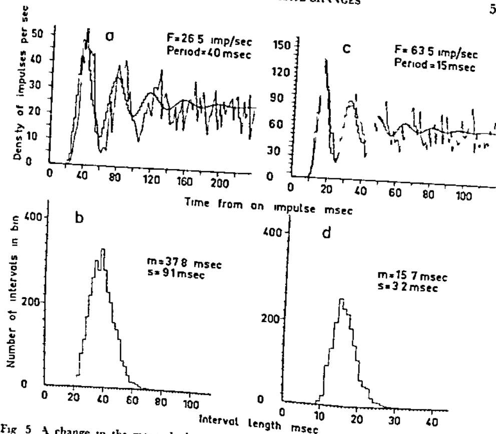


Fig 5 A change in the mean discharge rate accompanied by a parallel change in the frequency of the periodicity in a renewal process in cell 47101 (Fig 1 c) No change in the form of the interval histogram

before and after the rate change In the cell illustrated in Fig 6 the change in mean discharge rate is accompanied by a similar change in the frequency of the probability oscillations but in this cell the slower activity discloses a renewal process whereas the faster activity indicates a time dependent process In the faster activity the periodicity is enhanced apparently by a self-correcting firing mechanism in which too long an interval is followed by another correspondingly shorter to achieve a less variable firing time than the spread of the interval histogram would suggest

Discussion

In this material the standard deviation of the impulse intervals is a uniform function of the mean interval The same relation holds for samples from different cells as well as for different samples from the same cell The exponent of this function is 1 42 and the increase in the standard deviation values is thus steeper than t relation described by Werner and Mountcastle (1963) for driven activity

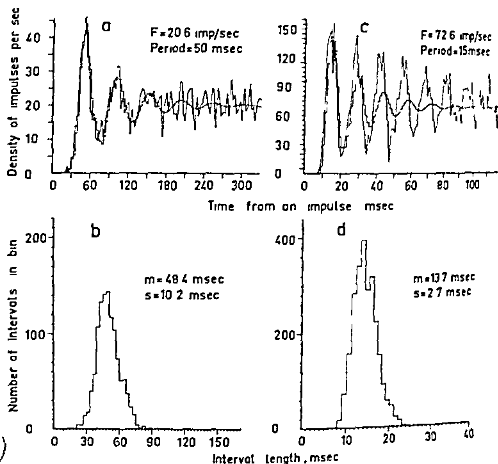


Fig 6 A change from a periodic renewal process to a periodic time dependent process in cel 44402 (Fig 1 d) No change in the form of the interval histogram Frequency of the period ty in the intensity function parallels the mean discharge rate

cells responding to joint position in unanesthetized monkeys In their material the mean coefficient of variation of the impulse intervals in driven activity was 0 52 whereas a different value 0 93 held for spontaneous activity independent of the difference in mean rates between driven and spontaneous activity In this material the coefficients of variation for spontaneous activity range from 0 20 in fast activity to 1 30 in slow activity Thus in the slow range of spontaneous activity the coefficients of variation were close to those described for spontaneous activity in unanesthetized monkeys and in fast activity the values were in the range described for driven activity As the change from low values to high ones was smooth it was not possible to use the coefficients of variation to judge whether the cell was under a specific drive or not

Although the steep increase of the coefficient of variation with increasing mean interval is partly caused by the change in the form of the distribution of intervals which tends to become more skewed with slower activity this increase does indic

a disproportionate irregularity of slow activity compared with the regularity of fast activity The increase in the dispersion is so steep that the exponent of the geometric relationship of standard deviation to mean is larger than one As the dispersion is not constant nor a constant proportion of the mean interval there is an actual difference between slow and fast activity in the mechanism that generates the dispersion

Although the observed rate changes were often quite large simultaneous changes were minor in the interval histograms and intensity functions Rodieck (1967) reported similar results for the spontaneous rate changes in cat retinal ganglion cells Also when recording from secondary olfactory cells under continuous stimuli Døving and Hyvarinen (1969) found only small variations in the structure of the impulse sequences at times of conspicuous mean frequency changes Poggio and Viernstein (1964) found changes in the frequency of the periodicity of expectation densities (intensity functions) in thalamic neurons responding continuously to joint position, these changes paralleled the changes in joint position and mean frequency of firing In 3 of our cells which had rapid discharge rates changes in mean frequency of firing were accompanied by similar changes in the frequency of oscillations in the intensity functions

The present results show that the stochastic properties do not provide reliable clues to the role of the different levels of spontaneous activity In the studies quoted above, analyses were made of the stochastic properties of the impulse sequences in responses to steadily maintained stimuli Those studies show that the stochastic properties of the impulse sequences either do not change with the mean discharge rate or change only *quantitatively in a way predictable if the change in the mean* discharge rate is known On the basis of our results and those of Rodieck 1967) the stochastic properties of the spontaneous activity are similarly linked to the mean discharge rate the only exception being the rhythmic periodicity found in some cells in slow spontaneous activity The role of the time structure of the spontaneous activity and activity in response to steady stimuli appears distinctly different from the role of the time structure found in responses to stimuli which vary in time like pure tones (Rose *et al* 1967 or cutaneous vibration (Mountcastle *et al* 1969) Because of synchronous activity in a population of cells responding to periodic stimuli the periodicity can be transmitted across several synapses whereas the time structure of spontaneous activity and steady response is likely to change at each synaptic level because of non synchronous convergence

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The Effect of Heparin and Some Related Agents on Diamine Oxidase in Rabbit Blood Plasma

By

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Abstract

HANSSON, R. and H. THYSELL. *The effect of heparin and some related agents on diamine oxidase in rabbit blood plasma.* Acta physiol. scand. 1970. *78.* 539—546.

In rabbits the blood plasma level of diamine oxidase (DAO) was followed after rapid i.v. injection of heparin, heparinoid substances, soluble ribonucleic acid and protamine chloride as well as the histamine releasing agents 48/80 and polymyxin B. Heparin(oid) injections elicited a striking DAO increase within 1/2 min, reaching maximal values within a little more than 1 hr and then smoothly declining to basal levels. The DAO increase was dose-dependent and seemed to be larger during pregnancy and in newborn animals. Continuous heparin infusion was followed by a delayed peak time and, in relation to the doses, a low but protracted increase in the blood plasma DAO. Sulfated polyanions caused a significant DAO increase, but no simple correlation was found between the size of the response and the amount of sulfur injected. The findings are discussed.

After i.v. injection of heparin into guinea pigs a transient marked rise of the blood plasma histaminase occurs (Bernauer *et al.* 1964, for a comprehensive discussion see Schmutzler 1966). It has been demonstrated, by this group, that the increase depends on a release of the enzyme in the guinea pig mainly from the liver. Independently it was found in man that i.v. injection of anticoagulant doses of heparin was followed by a rise of the diamine oxidase (DAO-) activity[1]—comparable to that found in pregnancy—in the blood plasma (Tryding 1965a, Hansson *et al.* 1966, Dahlbäck *et al.* 1968). Furthermore it has been shown, that the DAO increase in blood plasma after heparin injection is a ubiquitous finding throughout the vertebrate series investigated although a marked species variation occurred (Hansson and Thysell 1968). In guinea pigs the histaminase response in the blood plasma 2 min after the heparin injection is dose dependent, and this holds true also for other "polyanions"

[1] Footnote. Diamine oxidase (DAO) and histaminase are used synonymously in the text according to the Commission of Enzymes on the International Union of Biochemistry for no. 1.4.3.6. diamine: oxygen oxidoreductase (deaminating).

and "polycations" (Schmutzler *et al* 1966) As, however, this reaction had been studied only in a species with a supposedly unique reactivity among the vertebrates and as the reactivity pattern of blood plasma DAO in rabbit, according to our preliminary findings, seemed to resemble that in human beings after heparin we investigated in rabbits,

a) the dose response after rapid, slow and protracted intravenous administration of heparin,

b) the effects of some heparinoid substances and one sulfate free polyanion'

c) the effect of heparin on the blood plasma DAO during pregnancy and in new born animals

Material and methods

Material and methods

The animals drugs and other substances are described in Table I, together with doses and duration of injection/infusion The body weight of the non pregnant adult animals ranged between 1 9 and 3 0 (3 8) kg that of the pegnant animals was 3 0 and 3 3 kg The rabbits had free access to food pellets hay and water during experiments longer than 5 hrs When the duration of the experiments was shorter the animals were lying on their backs on the operation table if longer the animals were kept in cages with [illegible]
(Astra was used in 2—8 ml amoun
nulation polyethylene tubing int [illegible]
formed (with due consideration t [illegible]
new born animals where only the [illegible] calculated

The dose response group was arranged as a latin square (line 1 in Table I) [illegible] was re inforced with another 5 animals (line 2) each of which received different doses of heparin (0 0 0 2 0 4 0 8 and 1 6 U/g b w) the day before the standard dose 0 8 U/g which was interpolated between the doses 0 0 0 2 0 4 1 6 U heparin/g b w of group a and the completing injection of 5 µg Protamine® "equivalent to 0 4 U heparin per g b w [illegible]

In vitro the basal DAO levels from 8 rabbits [illegible]
of aminoguanidine to 10^{-3} M The DAO activi[illegible]
1965) ultramicro-modification of the ^{14}C putr[illegible]
and is outlined elsewhere Tryding and Willert 1968)

Results and primary conclusions

The individual changes in rabbit blood plasma diamine oxidase (DAO)-activity after heparin in different doses to new born (individual curves) pregnant and other adult animals mean values of each are depicted in Fig 1 As a background the plasma enzyme levels after i v injection of physiological saline and protamine are also included The basal DAO level in blood plasma varied about 0 02 U/l (S D – 0 006 if a normal distribution may be assumed Aminoguanidine inhibited basal values ranged between 0 000 and 0 007 with a mean ~0 003 This finding does not exclude that the determination of basal levels of DAO in blood plasma may be influenced by some minor extraneous factors In samples with about 2 U DAO/l there was however a 100 % inhibition with aminoguanidine of the same concentration 10^{-3} M in the incubation mixture Though not included in Table II there was a significant increase of the blood plasma DAO activity even after a half minute (0 12 U/l with a range of 0 06—0 23 when 0 8 heparin g was given against the basal level 0 02) In most experiments especially when the number of blood samples was

TABLE I Material and methods Except when otherwise noted, all dilution was in "saline" solution

Drug or substance	Relative amount U/g or μg g body wt	Number of animals	Number of experiments	Duration of injection (sec)	Comments
Heparin®, Vitrum, 5000 IU/ml in water, cont 0 2 % tricresol	0 2, 0 4 and 1 6	6*	16*	<2	
	0 8	5**	5**	<2	
	1 6	3	3	<2	Pregnant
	1 6	2	2	<2	16 hrs old
	1 6	2	2	120	Diluted in 20 ml.
	1 6	2	2		Diluted in blood, 20 ml
	1 6/hr during >24 hrs	2	2	> 10'	
Protamin®, Vitrum, 1 % (titrated to pH 7 0 with HCl)	5 μg	5*	5*	<2	
Physiol saline (9 g NaCl/l)	4 5 μg	5*	5*	<2	
Heparinoid substances					
G 31150, 10 %, Geigy	4 0, 5 0, 16 μg	2	6	<2	
Dermatan sulfate, 10 %, prepn from human aorta, Antonopoulos, Gardell and Hamnström 1965)	40, 100 μg	2	2	<2	Gift from Prof Sven Gardell, M D
Dextran sulfate ("Dextrarine"), l Equilibre Biologique, 10 %	12 μg	2	2	<2	
Histamine releasing agents					
Polymyxin B®, Novo	18 or 25 U	2	2	<2	The 18 U/g rabbit died in 5
48/80 Leo	0 3 and 1 2 μg	3	3	<2	The 1 2 ug/g rabbit died in 1'
Miscellaneous					
Soluble ribonucleic acid, type III from yeast Sigma	31 μg	1	1	<2	
Putrescine dihydrochloride, Sigma	48 μg	1	1	<2	
Trisodiumcitrate	1 μmole	2	2	<2	

* Rabbits belonging to the "latin square"

** "Interpolated" rabbits

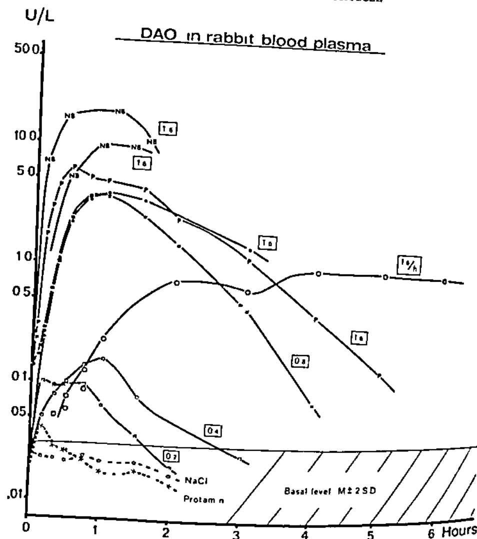


Fig 1 The diamine oxidase activity of rabbit blood plasma after injection—or infusion (1.6/h)—of heparin, sodium chloride and protamine chloride in isotonic solution given at time zero The framed numbers refer to the amount of heparin in units per g b w /h = per hour (in a constant rate infusion) P symbolizes pregnant and NB newborn animal(s)

large enough—the curve was biphasic (particularly after small doses of heparin) with two maxima or to a "shelf" before the culmination The DAO peak time also varied When 0.2 U heparin per g b w had been injected a maximum could be found as early as 10 or as late as 60 min after the injection (Because of these individual variations, the biphasic appearance is blurred) In the large-dose groups there was, however, a tendency to a more defined maximum about 60 min after the i v heparin administration

As a rule, each individual in the "latin square" arrangement responded more strongly when the heparin dose was increased The small number of observations

TABLE II Diamine oxidase activity (in U/l) in rabbit blood plasma The effect of intravenous injection of various agents (Duration of injection 2 seconds)

Agent	Dose per g body weight	Time after injection (minutes)								
		0	10	20	30	45	60	90	120	180
HEPARINOIDS										
1 G 31150	4 μg	016	081	22	45	54	55	36	17	043
	5 μg	019	44	47	64	> 52	52	16	052	021
	16 μg	012	28	1 36	1 60	1 59	1 66	1 48	85	31
2 Dextransulfate	12 μg	012	061	15	15	22	27	20	11	041
3 Dermatan	40 μg	035	033	055?	050	047	056	053	046	—
sulfate	100 μg	010	038	11?	16	33	30	36	33	13
SULFATE FREE POLYANION										
S RNA	31 μg	020	012	013	011	010	010	010	013	008
HISTAMINE RELEASING AGENTS	18 U	016	convulsions and death within 3 minutes							—
Polymyxin B	25 U	005	006	005	008	005	006	005	004	—
48/80	3 μg	013	011	—	033	—	009	011	008	008
	1 2 μg	012	convulsions and death within 2 minutes							
MISCELLANEOUS										
Putrescine	48 μg	012	006	008	009	007	010	—	009	—
Trisodium citrate	1 μmole	008	006	007	007	006	009	—	—	—

does not, however, allow conclusions regarding influences from i v heparin injections on the day before or other variations from day to day The same holds true also for the possible increase of blood plasma DAO after protamine in 2 out of 5 animals All of the 3 pregnant rabbits showed a maximal DAO activity about 30 min after the heparin injection whereas—after the same dose of heparin—in the other adult rabbits (vide supra) this occurred later

With increasing doses of heparin the average enzyme response tends to be stronger both as regards the maximum and the integral of the enzyme activity In both of these respects the 3 pregnant rabbits seem to give a rather more accentuated response to 1 6 U heparin/g than the other adult rabbits The few data available from the new born rabbits indicate that their DAO response to i v heparin may be even more pronounced than in the adult groups

When the i v administration of heparin was slower—2 min instead of 2 sec—no delay of the peak time could be noted Although the DAO activities recorded seemed to be low (mean value of $DAO_{max} = 2\ 0$ U/l) they did not differ materially from some of the corresponding dose curves in the other group Nor did mixing of heparin with blood before the injection seem to affect its DAO-effect, (mean values $DAO_{max} = 3\ 0$ U/l) as compared to dilution of heparin with "physiological

When heparin was infused at a rate of 1 6 U/g *per hour*, (Fig 1) there was a marked delay of the DAO peak time to between 5 and 8 hrs after the start of the infusion. The peak values (0 49 and 1 60 U/l, respectively) were also lower than all of the maximal DAO activities in the group of rabbits given 1 6 and 0 8 U/g as a rapid single dose in spite of the fact that the total amounts of infused heparin were at the peak time, more than 4 and 8 times larger respectively On the other hand the slowly declining DAO activity remained several times higher than the basal values, during the infusion period of more than 24 hrs

The rapid i v injection of the heparinoid substances G 31150 (Geigy) dextran sulfate and dermatan sulfate in essential details simulated the changes of the blood plasma DAO activity which were evoked by heparin (Table I) In contrast a barely perceptible no effect on the blood plasma DAO was noted after injection of protamine and no response to soluble ribonucleic acid trisodium citrate putrescine or the histamine releasing agents polymyxin B or 48/80 (Table II) could be traced

Discussion

In vitro addition of heparin to guinea pig blood (Bernauer *et al* 1964) or to human blood plasma (Tryding 1965) does not activate histaminase/diamine oxidase This finding seems to hold true also in other vertebrate species (our findings)

The intravenous injection of heparin is on the other hand followed by a significant increase of the blood plasma diamine oxidase (DAO) activity varying in size peak time and duration between different vertebrate species investigated but a constant finding in all of them (Hansson and Thysell 1968) Very little is known of the DAO releasing mechanisms except for the triggering effect of anaphylactic shock in some species and of heparin(oids) A recent communication (Schmutzler *et al* 1968) however reports that in the guinea pig liver tissue slices do not release histaminase in excess when incubated with heparin *in vitro* whereas the (exsanguinated) organ will liberate the enzyme when perfused with blood free heparin solution Similar studies on isolated organs in rabbit or other species do not seem to have been published as yet

Our present findings strongly indicate that there is a dose dependent heparin induced increase of the blood plasma DAO in rabbits This increased enzyme activity starts within one minute but has a later maximum than in the guinea pig (cf Schmutzler 1966 and Hansson and Thysell 1968) and its peak size is after the larger heparin doses only about one fifth of that in the latter species Finally the peak response to rather small heparin doses (0 2 U/g) in the guinea pig is still very high whereas our rabbits when given the same relative doses of heparin in a few cases, gave a hardly significant elevation of the DAO over the basal level Such differences may explain earlier reports that no histaminase liberation occurred after heparin injection into rabbits (or rats dog fowl or man cf Schmutzler 1966) The possible identity of histamine with diamine oxidase is an unsettled problem but as to amino-guanidine sensitivity and pH-optimum the heparin induced DAO activity in

all speces examined is not different from the histaminase in pregnancy (Hansson, in press) In human pregnancy there is a close parallelism between the DAO determination with Tryding's ultra micromethod and Ahlmark's histaminase method (Tryding and Willert 1968)

During human pregnancy, the heparin injection is followed by a rather early and marked further rise of the blood plasma diamine oxidase as compared to non pregnant individuals, but the return to pregnant basal DAO levels occurs as in the latter group (Hansson, Tryding and Tornqvist 1969) In the rabbit where the placental histaminolytic effect per gram is only about 20 % of that found in the human placenta (Swanberg 1950) and where no demonstrable increase of the basal levels of the histaminase /diamine oxidase activity is found in blood plasma during pregnancy, there was still the same tendency to an early and powerful response of plasma DAO after i v heparin This suggests that the blood plasma DAO increase after heparin is derived from extrauterine structures also during pregnancy

The two new born rabbits reacted with a pronounced DAO increase in the blood plasma after i v heparin This finding agrees well with adult amounts of histaminase found in the small intestine of kitten but, seemingly contrasts to the possibility that the enzyme is liberated from an organ that has a postnatal build up period of this enzyme as has been reported on the kitten kidney (*cf* Haeger Kahlson and Westling 1953)

Very little is, however, known of the distribution of DAO in the organs of the rabbit and its ontogenetic variation

The finding that i v injection of different other sulfated "polyanions" will liberate histaminase in the guinea pig has been thoroughly investigated (Schmutzler 1966) Our fragmentary experiments indicate that this effect is not unique for the guinea pig Whereas some dose dependence is suggested no simple correlation can be found between the amount of sulfur in the heparinoid substance and the DAO response

In guinea pigs the i v injection of protamine (clupeine) is followed by a pronounced rise of the blood plasma histaminase, if more than 2 μg/g body weight are given (Schmutzler 1966) Although the protamine dose to our rabbits was 5 μg/g body weight the effect—if any—was very poor Nor could any increase of the blood plasma DAO activity be seen after the two basic histamine releasing agents polymyxin B and compound 48/80 even in toxic doses It is however, premature to discuss the species difference such as the possibilities of substrate competition *in vitro* heparin neutralization or no heparin release *in vivo*, against the background of the present knowledge of the multifaceted problems encountered in the study of the basic histamine releasing compunds (for ref see Rothschild 1966)

During anaphylactic shock the blood plasma histamine-destroying capacity/histaminase is increased in some species (Rose and Leger 1952 Logan 1961 Code *et al* 1961) The mediating action of heparin during the release of this enzyme has been established in the guinea pig (for references see Hahn and Schmutzler 1967 Giertz *et al* 1968) In this context it is of interest to note that, from birth o

possibly intensified during pregnancy, the same reactive pattern after i v injection of heparin exists in the rabbit as in the guinea-pig However, quantitative species differences exist, just as there seems to do throughout the series of (adult) vertebrates (Hansson and Thysell 1968)

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Acta physiol. scand 1970. 78 547—560

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The Release of Histamine, Heparin and Granule Protein from Rat Mast Cells Treated with Compound 48 80 in vitro

By

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Abstract

FILLION G M B S A. SLORACH and B UVNAS *The release of histamine [illegible] and granule protein from rat mast cells treated with compound 48/80 in vitro* Acta physiol scand 1970 78 547—560

The quantitative relationship between the release of histamine heparin and granule protein from rat mast cells expo ed to compound 48/80 *in vitro* has been investigated in order to clarify the mechanism of action of this releaser A correlation was found between the three release curves The ratio of heparin to granule protein was similar in extruded granules and [illegible]

[illegible] cations in the extracellular medium

When isolated rat mast cells are exposed to compound 48/80 extrusion of granules can be observed and release of histamine takes place In such cells histamine is stored in discrete intracellular granules which mainly consist of a protein heparin complex Aborg Novotny and Uvnas (1967) have presented evidence that in isolated granules histamine is electrostatically linked to carboxyl groups of the complex. It is released by suspending the granules in cation containing media (Uvnas 1964) It is probable that histamine is bound to the granules in a similar way in situ [illegible] volved in [illegible] presented

(Uvnas and Thon 1965 [illegible]) that the response of mast cells to compound 48/80 can be divided into two stages The initial event the degranulation process is the result of the triggering of an energy requiring transport of histamine-containing granules to the outside of the cell The second phase is an extracellular exchange between histamine in the extruded granules and [illegible]

the extracellular medium If this hypothesis is correct and an extrusion of granules precedes the release of histamine, there should be a correlation between the amounts of histamine and granules released assuming that the histamine within the cells is evenly distributed with respect to the granules

In order to examine the validity of this hypothesis we have studied the release of histamine, heparin and granule protein from rat mast cells exposed to compound 48/80 *in vitro* From the results obtained in these studies it was also possible to calculate the amounts of heparin granule protein and histamine in normal rat mast cells

Methods and materials

Isolation of mast cells

Mast cells were obtained from the peritoneal and pleural cavities of male Sprague Dawley rats (350—400 g) and separated from other cells by density gradient centrifugation in Ficoll ac ... The cells were counted in a Burker chamber

... lled cells were required the rats were injected ... the cells. This yielded cells giving ca 40 000

Localization of ^{35}S *in mast cells*

^{35}S labelled mast cells obtained from four rats were suspended in 6 ml of isotonic sodium ... The supernatant which ...

The first 2 ml of eluate was disc... of eluate had been collected and ... in both cases This was followed ... and finally with 20 ml of 4 M N... minute throughout Each fraction was assayed for

a) Protein — by measuring the absorption at 230 mμ

b) Heparin — by the method described below using 0 2 ml samples of each fraction diluted with 0 2 ml of water or 0 4 ml samples (aq dist eluates)

c) Radioactivity (^{35}S) — by the method described below using 0 5 ml samples of the distilled water 1 M and 4 M NaCl eluates and 0 1 ml samples of the 2 M NaCl eluates

Fig 1 shows the result of an experiment in which mast cells from $Na_2^{35}SO_4$ treated rats were subjected to chromatography ... no apparent similarities inc... have been separated The el... 3—11 inclusive of the 2 M ... the total ^{35}S and the amount of radioactivity outside the heparin peak was ... of the Dowex column after elution did not show any radioactivity It seems justifiable to conclude that the ^{35}S in the mast cells is almost exclusively present in heparin molecules

Incubation and washing procedures

a) *General method*

Isolated ^{35}S labelled mast cells ... salt solution NaCl 145 mM, KCl 2 7 mM CaCl₂ 0 9 mM ... buffer (Na_2HPO_4 + KH_2PO_4 67 mM) pH 6 9 and ... incubations were carried out in a volume of 1 ml in ... with tightly fitting stoppers Compound 48/80 was added directly to the cell suspension ... immediately prior to incubation at 37° C for 10 min

After incubation, the tubes were centrifuged (350 × g 10 min) and the supernatants were decanted The cells were then washed by resuspending them in 2 ml of salt solution (vide supra)

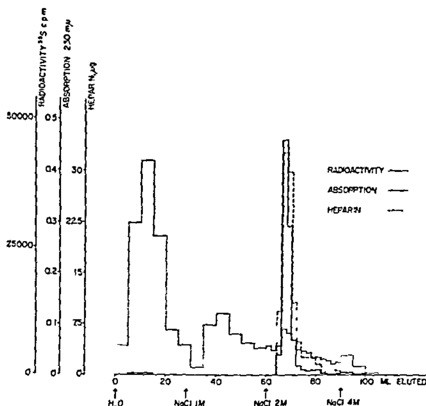


Fig 1 Chromatographic separation of ³⁵S labelled mast cell components on Dowex 1—X2 The absorption at 230 mμ is used as a measure of the protein eluted

containing human serum albumin 1 mg/ml and buffered to pH 7 8 centrifuging as above and decanting the supernatant Three 2 ml washes were carried out in this way giving a total $350 \times g$ supernatant volume of 7 ml In order to minimize the spontaneous release of histamine all procedures after the incubation were carried out at 4° C After three washes the cells were disrupted by suspending them in 2 ml of distilled water The radioactivity (^{35}S) in 0 5 ml samples of each $350 \times g$ supernatant and in 0 1 ml samples of the disrupted cell suspension was determined as described below

b) *Effect of pH of the washing fluid*

(1) microscopical observations

Mast cells were incubated with compound 48/80 (1 μg/ml) and washed as described in the general procedure (*vide supra*) except that the washing solution was adjusted to pH 6 4 6 9 7 2 7 3 7 4 or 7 8 or was adjusted to pH 6 4 or 7 6 and albumin free After each wash the cells were examined under the light microscope

(2) *^{35}S determinations*

^{35}S-labelled mast cells were incubated in the presence or absence of compound 48/80 (1 μg/ml) and washed as described under the general procedure except that four 2 ml washes were used In parallel with this a similar experiment was performed using washes adjusted to pH 6 9 The levels of radioactivity in the different fractions were determined as in the general procedure

the extracellular medium If this hypothesis is correct and an extrusion of granules precedes the release of histamine, there should be a correlation between the amounts of histamine and granules released, assuming that the histamine within the cells is evenly distributed with respect to the granules

In order to examine the validity of this hypothesis we have studied the release of histamine, heparin and granule protein from rat mast cells exposed to compound 48/80 *in vitro* From the results obtained in these studies, it was also possible to calculate the amounts of heparin granule protein and histamine in normal rat mast cells

Methods and materials

Isolation of mast cells

with 4 mCi Na_2 $^{35}SO_4$ s c two days before taking the cells This yielded cells giving —70,000 cpm per 10^6 cells

Localization of ^{35}S in mast cells

^{35}S-labelled mast cells obtained from four rats were suspended in 6 ml of isotonic sodium chloride buffered to pH 6 9 and then centrifuged at $350 \times g$ for 10 min The supernatant which contained a negligible amount of radioactivity was discarded and the cells were dissolved in 2 ml of 0 05 N NaOH 8 ml of 1 M NaCl was added and the solution was adjusted to pH 6 9 with 0 1 N HCl 10 ml of this solution was added to a Dowex 1 X2 column (10×200 mm) ... until 130 ml ... were collected ... ml fractions ... 0 5 ml per

a) Protein — by measuring the absorption at 230 mμ

b) Heparin — by the method described below using 0 2 ml samples of each fraction diluted with 0 2 ml of water or 0 4 ml samples (aq dist eluates

c) Radioactivity (^{35}S — by the method described below using 0 5 ml samples of the distilled water 1 M and 4 M NaCl eluates and 0 1 ml samples of the 2 M NaCl eluates

Fig 1 shows the result of ... were subjected to chromatography ... no apparent similarities inc ... have been separated The el ... of 3—11 inclusive of the 2 M ... the total ^{35}S and the amount of radioactivity outside the heparin peak was ... of the Dowex column after elution did not show any radioactivity It seems justifiable to conclude that the ^{35}S in the mast cells is almost exclusively present in heparin molecules

Incubation and washing procedures

a) *General method*

Isolated ^{35}S-labelled mast cells 1 1—1 9 ... salt solution NaCl 145 mM KCl 2 7 mM $CaCl_2$ 0 9 mM conta ... HPO_4 + KH_2PO_4 67 mM) pH 6 9 and 1 mg ... were ... fitting ... ml mg of ... prior to

50×g 10 min) and the supernatants were ... ng them in 2 ml of salt solution (vide supra)

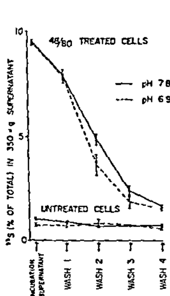


Fig 3

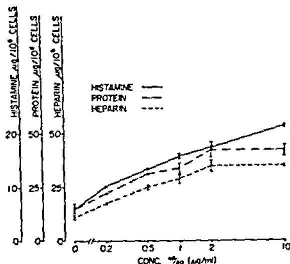


Fig 4

Fig 3 Removal of ^{35}S-labelled granules from untreated and compound 48/80 (1 μg/ml)-treated mast cells by repeated washing in buffered salt solutions at pH 6 9 or 7 8 Each point is the mean of duplicates, vertical bars indicate range

Fig 4 Release of histamine, granule protein and heparin from mast cells treated with increasing doses of compound 48/80 Each point is the mean of duplicates, vertical bars indicate range

outside of the cells, this is in accordance with the observation of Thon and Lynas (1966) However, some clumps of granules and grossly damaged cells, which are precipitated by centrifugation at 350 × g were observed

The radioactivity (^{35}S) in the different 350 × g supernatants from untreated mast cells and from mast cells treated with compound 48/80 (1 μg/ml) and washed at pH 6 9 or pH 7 8 is shown in Fig 3 It can be seen that there is little advantage in using 4 washes instead of 3 [illegible] total [illegible] third [illegible] reated [illegible] 6 3 % [illegible] 3 7 % respectively [illegible] general procedure

Release of histamine ^{35}S (heparin) and granule protein on exposure to compound 48/80

Mast cells labelled with ^{35}S were incubated alone or with compound 48/80 (0 2—10 μg/ml) and washed as described under the general method The combined 350 × g supernatants (total 7 ml) and the disrupted cell suspensions were assayed for histamine and ^{35}S The granules [illegible]

et al (1951) (*vide infra*) The protein in the second granule wash was determined in order to check that the granules had been freed from albumin originally present in the 350 × *g* supernatant

– ^{35}S (cpm) and heparin (μg) was established and histamine assays, the disrupted cell sus-

assayed for radioactivity and heparin

Calculation of results

percentage

cells and

he 350 × *g*

supernatant is used as a measure of the ^{35}S released in the granules

Heparin — from the relationship between ^{35}S (cpm) and heparin (μg), measured as described above, the release of heparin could be calculated from the ^{35}S results

Protein — since the granules are practically insoluble in water (Thon and Uvnäs 1966) it ... and ^{35}S between the 350 × *g* supernatant and the multiplying the protein in the washed granules washed granules the amount of granule protein tained A correction was also applied to take account of the fact that the granules were recovered from only 5 8 ml of the 7 ml total 350 × *g* supernatant

Protein, ^{35}S and heparin in mast cell granules isolated in different ways

Granules were obtained from ^{35}S labelled mast cells by the following procedures

(a) Cells were treated with compound 48/80 (1 μg/ml) and the extruded granules were recovered as described above

(b) ... (a) were lysed with distilled water and, aft... er particles the granules were precipitated ... twice with distilled water

(c) ... and the granules were collected as describe... of the samples in some cases the ratio ^{35}S/heparin was also determined

Release of histamine and ^{35}S from frozen and thawed mast cells

^{35}S labelled mast cells were frozen and thawed once or twice in albumin-containing salt solution pH 6 9 (*vide supra*) and then washed according to the general method The percentages of histamine and ^{35}S released into the 350 × *g* supernatant were determined

Extraction of ^{35}S from mast cells by freezing and thawing in 0 1 N hydrochloric acid

^{35}S labelled mast cells were frozen and thawed 3 times in 0 1 N HCl and then centrifuged (2700 × *g*, 20 min) The ratio radioactivity in the supernatant/total radioactivity was then determined

Liquid scintillation counting

After dissolution in 15 ml of scintillator fluid, the radioactivity in the various samples was determined using a Tri Carb liquid scintillation spectrometer Packard Instrument Co, Inc., La Grange, Ill, U S A The scintillator fluid consisted of a solution of 0 4 %, 2 5-diphenyloxazole and 0 01 % 1,4-bis 2-(4 methyl 5 phenyloxazolyl) benzene in a mixture of equal volumes of toluene and ethylene glycol monoet... been made for background radioactivity (~35 cpm) ... (>63 %), since these factors did not influence c... practically unchanged by the addition of 0 05 N

Histamine assay

Histamine was measured flurometrically according to the method of Shore Burkhalter and Cohn (1959), but since isolated mast cells do not contain any substances which interfere with

the histamine assay (Bergendorff to be published) the purification steps were omitted and the o-phthalaldehyde was added directly to the samples after alkalinization All histamine values refer to the free base

Protein assay

Protein was assayed by the method of Lowry *et al* (1951) and the results are expressed in terms of the crystalline pepsin standard used

Heparin assay

Heparin was determined using the method of Glick von Redlich and Damant (1967) with the modifications that the procedure was carried out on a larger (10×) scale and a Zeiss spectrophotometer Model PMQ II with monochromator M40 III and 1 cm cuvettes were used. According to Jaques (1961) rat heparin contains 13 7 % sulphur The heparin used in our experiments contained 8 99 % sulphur and the values obtained were converted to and are expressed as rat heparin using the factor 0 656

Materials

Ficoll was obtained from AB Pharmacia Uppsala Sweden pepsin crystallised twice from dilute alcohol from Mann Research Labs Inc U S A and human serum albumin (free from preservatives) from AB Kabi Strangnas Sweden. Compound 48/80 was kindly supplied by Dr B Hogberg AB Leo Halsingborg Sweden Sulphur 35 as carrier free sulphate in sterilized neutral isotonic solution was obtained from the Institutt for Atomenergi Kjeller Norway and heparin sodium (p g mucous) containing 8 99 % S from AB Vitrum Stockholm Sweden All other materials were obtained from normal commercial sources

Results

Release of histamine, heparin (^{35}S), and granule protein

a) *Compound 48/80*

The release of histamine heparin and granule protein in an experiment in which mast cells were incubated with various concentrations of compound 48/80 is shown in Fig 4 The release curves are fairly parallel especially those of heparin and protein A similar relationship was obtained in a further three experiments although the absolute amounts released were different due to variations in the concentration of cells in the incubation medium and consequent differences in the response to compound 48/80

The loss of granules (measured as ^{35}S loss) during the granule isolation and washing procedures was less than 20 % The second granule wash was normally virtually free from protein When small amounts of protein were detected this was due to mechanical transfer of granules during decanting of the second wash and in these cases the wash contained a corresponding amount of ^{35}S

Fig 5 shows the release of histamine and ^{35}S expressed as percentages of the total cell content in the experiment illustrated in Fig 4 There is a clear correlation between the two curves and this was so for all five experiments performed

The ratio percentage histamine release/percentage ^{35}S release in 5 expts is shown in Fig 6 For mast cells treated with compound 48/80 (0 2—10 μg/ml) the ratio is about 2 2 at all concentrations although for untreated cells the mean ratio is a little higher the variations are too great for this to be significant The ratio 2 2 is artificially high due to a number of experimental artefacts

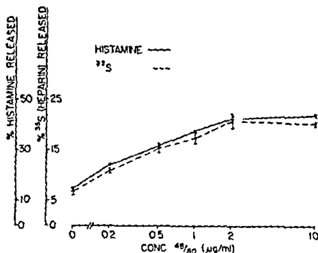


Fig 5 Release of histamine and 35S expressed as percentages of the total cell content from mast cells treated with compound 48/80 in vitro Each point is the mean of duplicates vertical bars indicate range

First of all some of the cells seem to be damaged after the washing procedures In order to estimate the proportion of damaged cells 5×200 compound 48/80 treated (1 µg/ml) and washed cells were inspected under the light microscope (1000× magnification) Cells were classed as damaged when the membrane appeared disrupted The proportion of damaged cells (mean ± S D) found was 16 2 ± 1 5 % These cells will probably lose proportionally more of their histamine than their granules A further correction should be made to take account of granules not collected due to adhesion to the cells or clumping From microscopical observations we estimated that the granules remaining attached to the cells did not exceed 15 % of the total number of granules released

Considering the case of cells exposed to compound 48/80 1 µg/ml (Fig 5 Histamine release 38 % ^{35}S release = 17 5 % Ratio 2 2) then correcting for the assumed maximum 15 % of extruded granules not collected would reduce the ratio to a minimum of 1 8 The correction for the damaged cells is impossible to quantitate since the relative amounts of histamine and granules lost by such damaged cells is not known However such a correction would further reduce the ratio probably to somewhere in the range — 1 3 — 1 7

b) *Freezing and thawing*

In two experiments in which ^{35}S labelled mast cells were frozen and thawed once in the solution used for incubation and then washed using the general procedure the release of histamine was 99 % (both cases) and the release of ^{35}S was 41 % and 42 % respectively If the freezing and thawing was repeated all the histamine and 49 % of the ^{35}S was released

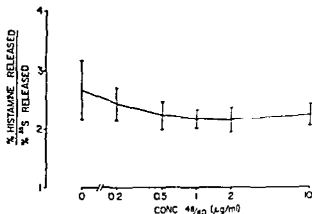


Fig 6 Ratio Percentage histamine release/Percentage ^{35}S release from mast cells treated with compound 48/80 *in vitro* Each point represents the mean of 5 expts. in each of which duplicate samples were taken at each concentration Vertical bars indicate S D

Extraction of heparin by freezing and thawing

Freezing and thawing 1 3×10⁶ or 1 1×10⁶ ^{35}S labelled mast cells in 2 ml of 0 1 N HCl extracted 13 % and 21 % respectively of the ^{35}S (and therefore heparin into the supernatant

Cell content of histamine and heparin

The mast cell contents (μg per 10⁶ cells mean ± S D) of histamine and heparin in 5 expts were 34 1 ± 6 1 and 104 5 + 38 7 respectively The ratio heparin/histamine was 3 0 ± 0 65

The values obtained for histamine content are similar to those reported by Glick von Redlich and Diamant (1967) for mast cells from the same strain of rats However, our heparin values, and consequently heparin/histamine ratios are almost 3 times those obtained by the above authors In our experiments we found that the extraction of ^{35}S (heparin) from mast cells by repeated freezing and thawing in 0 1 N HCl was very incomplete It is probable that the poor extraction of heparin by this method, used by Glick von Redlich and Diamant (1967) accounts for the low values for heparin content they obtained In our method the poor extraction is of no importance since the heparin values are calculated from the ^{35}S/heparin ratio in the extract and the ^{35}S content of the cells Bergendorff (to be published) and Benditt, Arase and Roeper (1956) found the ratio of heparin to histamine in isolated rat mast cells to be 3 6 and 3 respectively The results of both these studies in which the heparin was measured by methods different from ours are in good agreement with our findings

^{35}S/Protein and Protein/Heparin ratios

Table I shows the ratios of ^{35}S/protein and protein/heparin in a) granules extr from cells exposed to compound 48/80 (1 μg/ml), b) granules remain

TABLE I

Expt	Granule Isolation Procedure	^{35}S (cpm/ml) / Protein (µg/ml)	Protein (µg/ml) / Heparin (µg/ml)	Heparin content (µg/10⁶ cells)	Granule protein content (µg/10⁶ cells)
1	a	478	1.24		
	b	480	1.24	125	159
	c	444	1.34		
2	a	285	1.13		
	b	286	1.13	137	166
	c	234	1.37		
3	a	419			
	b	467			
	c	394			
4	a	250			
	b	259			
	c	29[illegible]			

a) Granules extruded from cells treated with compound 48/80 (1 µg/ml)
b) Granules obtained by water lysis of compound 48/80 treated and washed cells
c) Granules obtained by water lysis of untreated cells

after degranulation and c) granules from untreated cells. In the latter two cases the granules were obtained by lysing the cells with water. The ratios are similar within each experiment, regardless of the method of isolating the granules. The fact that the numerical values of the ^{35}S/protein ratios differ between experiments *is of no significance* since the granules were isolated from different batches of cells in which the ^{35}S/heparin ratios were different due to variations in the amount of ^{35}S incorporated into the granule heparin.

The ratio of *protein (pepsin) to rat heparin in mast cell granules* obtained in the release experiments (*vide supra*) was similar within each experiment but considerable variation between experiments was found (1.05, 1.25, 2.30, 2.32 respectively). In the experiments where the ratio was high the heparin contents per million cells were particularly low. The granule protein content per million mast cells determined from both the release experiments and the experiments shown in Table I was found to be 187.1 ± 32.5 µg (mean of 6 expts ± S.D.).

Thus the total weight of the cell components histamine, heparin and granule protein will be 325.9 µg. Diamant and Lowry (1966) found the average dry weight of rat peritoneal mast cells to be 476 µg per million cells. If the weight of our cells was similar and neglecting any differences which may arise through the use of different methods for determining protein, then the three components we have studied (histamine, heparin and granule protein) would represent 68.5 % of the total weight of the cell.

Discussion

Jorpes Odeblad and Bostrom (1953) and Lagunoff, Calhoun and Benditt (1960) showed that the maximum uptake of ^{35}S into rat mast cell heparin occurred after 48 hrs and the level decreased slowly thereafter We allowed this period between injection of the animals and isolation of the mast cells Green and Day 1960) found that when neoplastic mouse mast cells were incubated in $^{35}SO_4$ after 16 hrs 95 % of the isotope in the cells was present as heparin The heparin protein complex of the mast cell granules dissociates in strongly alkaline solution or in concentrated salt solutions (Bergqvist Samuelsson and Uvnas to be published) Thus by dissolving the cells in sodium hydroxide and adding sodium chloride solution and neutralizing followed by chromatography on a Dowex 1 X2 column we were able to separate the heparin from the protein components of the cell It was found that the elution of heparin and ^{35}S occurred in the same fractions indicating that virtually all the ^{35}S is localized in heparin molecules Lagunoff and Benditt (1963) state that more than 91 % of the heparin in mast cells is located in the granules Therefore a study of the ^{35}S release will provide useful information about the release of granules provided that it can be shown that granule protein is released together with the heparin and provided that the ^{35}S labelled heparin is evenly distributed with respect to the granules

The ^{35}S/protein ratios in extruded granules granules remaining in cells after degranulation and granules from untreated cells were similar This indicates that the ^{35}S labelled heparin was evenly distributed with respect to the granule protein and thus establishes the validity of using ^{35}S release as a measure of granule release

Uvnas (1964) put forward a hypothesis to explain the release of histamine from rat mast cells exposed to compound 48/80 He suggested that the release occurred as an *extracellular* cation exchange following extrusion of the histamine containing granules from the cells Under ideal conditions assuming that the histamine is evenly distributed with respect to the granules the release of a certain percentage of histamine should be accompanied by the release of the same percentage of granules *i e* the ratio percentage histamine release/percentage granule release should be unity In the following discussion we have assumed that the histamine and the ^{35}S heparin within the mast cells are evenly distributed with respect to the granules

In addition the granules remaining inside the cell should appear unchanged In order for these ideal conditions to be fulfilled it must be possible to separate the extruded granules completely *from the cells and the cells themselves must not be* damaged during the experimental procedures In practice when mast cells are exposed to compound 48/80 the extruded granules adhere to the cells giving them a mulberry like appearance (see Fig 2 b—2 c and Uvnas and Thon 1961) After washing the cells several times with buffered salt solution most of the adhering granules appear to have been removed (Fig 2 d 2 e) However the granules can also adhere to each other forming clumps which are precipitated together with the cells at 350×g In addition a proportion of the treated cells seem damaged after washing and although they were still precipitated with the whole cells th

membranes appeared grossly damaged which would enable a considerable proportion of the histamine to leak out

In this connection the experiments with the frozen and thawed cells show that even though the cells are extensively disrupted and release all their histamine it is impossible to recover all the granules with the technique used The granules adhere to each other and also to disrupted cell fragments This observation illustrates the difficulty in making quantitative correlations of histamine and granule release from intact compound 48/80 treated cells

In our experiments we have found that when mast cells were exposed to compound 48/80 the ratio percentage histamine release/percentage ^{35}S release was about 2 2 However, because of the experimental artefacts discussed above this ratio is artificially high Correcting for these artefacts will reduce the ratio probably to somewhere in the range 1 3—1 7 p 554) Thus our results agree with the assumption that following exposure to compound 48/80 at least the major part of the histamine release takes place in the manner proposed by Uvnas (1964) The remainder of the release appears to occur in such a way that no net loss of granules from the cells occurs This could take place by some kind of *intracellular* cation exchange Bloom and Haegermark (1965) showed that in mast cells exposed to compound 48/80 some of the granules appeared enlarged with reduced electron density and suggested that the alteration in the appearance of the granules was due to intracellular histamine release

If we assume this intracellular histamine release to be the result of a cation exchange in the granules — and no evidence exists to-day in favour of another release mechanism in the granules — there are two main possibilities to explain the release The first is an exchange between histamine and intracellular cations possibly potassium However there is no experimental evidence that compound 48/80 initiates the intracellular transport processes necessary *for intracellular* cation passage through the membrane surrounding the histamine carrying granules The other possibility is a transport of cations (*e g* Na from the extracellular milieu into the granules Slorach and Uvnas (*1969*) found that there was in fact an *uptake of sodium* into mast cells exposed to compound 48/80 although this uptake even if it had been into the granules could only account *for a minor* part of the observed histamine release

We believe it is possible to explain the compound 48/80 induced histamine release on the basis of a single mechanism It has been suggested that granules storing various biologically active substances (enzymes hormones) are stored *in intracellular* canaliculi from which they *are* expelled together with the active material Similarly — although it has never been conclusively shown — several authors (*e g* Padawer 1969 and Gillespie Levine and Malawista 1968) have proposed that *mast* cell granules lie in tubules or canaliculi running from the *surface of the cell* into its interior Vacuoles containing granules have been observed *in electron micrographs* of *mast* cells exposed to compound 48/80 *in vitro* (Horsfield 1965 and *Bloom* and Haegermark 1965) If it is assumed that in the mast cells the granules are *stored in*

such tubules it could be imagined that granules not expelled during the degranulation process but localized in tubules temporarily open to the ex r of the cell are exposed to the cation-containing extracellular medium and in r hu a release of histamine could occur The histamine could be released from all he g es in the tubule but all these granules may not be expelled before the tub e again Under these circumstances all the histamine could be released from tl nule b cation exchange for extracellular ions Whether the intratubular ca exchange should be described as extra or intra cellular is largely a semantic q Since the only granule membrane is the canalicular wall at the time of the r hange the granules will not be separated from the extracellular fluid by any m mbrane and thus it could justifiably be described as extracellular

Johnson and Moran (1969) state that release of histamine from rat mast cells induced by compound 48/80 was not accompanied by a release of protein but that selective release of granules takes place These two statements appear to be contradictory They based their conclusions on the fact that there was n reduction of the protein in the precipitated compound 48/80 treated cells after centrifugation They do not appear to have attempted to separate the extruded granules from the cells and their centrifugation procedure (1000 × g 10 min) may well precipitate most of the granules and therefore most of the granule protein together with the cells Their results do however show that little or no *soluble* protein is released from the cells by compound 48/80 treatment

Our results show that the release of histamine from mast cells in response to compound 48/80 involves the extrusion of granules i e a histamine carrying heparin protein complex During the extrusion process the histamine carrying granule leaves behind the membrane which surrounds it intracellularly (Horsfield 1965 Bloom and Haegermark 1965) In the adrenal medullary cell as well as in nerve terminals adrenaline and noradrenaline are stored in granules or vesicles together with another acid polymer ATP and a protein It was suggested from our laboratory (Uvnas and Thon 1965) that the mast cell response to compound 48/80 with the expulsion of the whole amine carrying heparin protein complex together with histamine might serve as a model for the release of catecholamines — and other amines — as induced by nerve stimulation from the adrenals and from nerve terminals It has now been demonstrated that the release of adrenaline from adrenal medullary cells is accompanied by an extrusion of the whole vesicle content ATP protein and adrenaline (*vide* Douglas 1968 and Schneider Smith and Winkler 1967) The similarity with the compound 48/80 induced mast cells response is obvious The mast cell response might also serve as a model for the processes behind the release of other granule bound biologically active substances It has been repeatedly demonstrated that the degranulation can be inhibited by metabolic inhibitors and other substances interfering with the aerobic or anaerobic metabolism of the mast cell It is easy to understand that the extrusion of the basophil granules — 0 1—0 5 μ in diameter — from the cells requires energy It is more difficult to imagine the intimate nature of this transport mechanism Some kind of a mechanical transport due to a water pu

system or a tubular contraction process seem to be attractive hypotheses worthy of future investigation (Thon and Uvnas, 1967, Gillespie, Levine and Malawista 1968)

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Facts and Artifacts in Gradient Centrifugation Analysis of Catecholamine Granules

by

H Lagercrantz, H Pertoft and L Stjärne

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Abstract

Lagercrantz, H, H Pertoft and L Stjärne *Facts and artifacts in gradient centrifugation analysis of catecholamine granules* Acta physiol scand 1970 *78* 561—566

Estimates of the "true" density of catecholamine (CA) storage granules from sympathetic nerves and adrenal medulla have previously been largely based on sucrose density gradient centrifugation In view of the danger of osmotically induced artifacts in this type of combined density and osmolarity gradient the present experiments were carried out to determine the sedimentation characteristics of different classes of CA granules in silica gradients of homogenous and adjustable tonicity In isotonic gradients chromaffin granules were found to be of abo[illegible] [illegible] meability to sucrose in these particles

Catecholamine (CA) granules from the adrenal medulla are generally regarded as relatively heavy since they sediment further in sucrose density gradient centrifugation than do mitochondria (Blaschko *et al* 1957, Potter and Axelrod 1963) By contrast nerve granules from tissues like the rat heart have been referred to as relatively light sedimenting to the level of microsomes in the same type of gradient (Potter and Axelrod 1962) In view of the similarities between nerve and medullary CA granules and also because of the common origin of sympathetic neurons and chromaffin cells these differences in apparent density are hardly to be expected

The issue is further complicated by the finding that nerve granules when centrifugated in continuous linear sucrose density gradients (Roth *et al* 1968) yield two different particulate populations one sedimenting to a level of 0 5 M sucrose and another to 1 2 M sucrose

The confusion concerning the true density of the granules might to some extent be due to changes of their original density induced by the hypertonicity of the sucrose gradients In order to test this possibility we have repeated the grad

centrifugation analysis of CA granules using a self-generating silica solution density gradient (Pertoft 1966). The pure silica solution has a low osmotic pressure. The density gradient formed depends on differences in size of the silica particles and the osmolarity can be adjusted at will by addition of sucrose.

Material and Methods

Density gradient centrifugation

The gradients were prepared according to Pertoft and Laurent (1969), using a silica solution commercially available under the trade name Ludox HS (DuPont de Nemours). In this solution the silica particle diameter ranges from 80 to 200 Å, and the average density is 1.30 g/cm³ (40 % solution in water w/w). It has a low viscosity and the osmolarity can be adjusted to the desired strength by addition of *e.g.* sucrose. On centrifugation of Ludox HS preferably in an angle head rotor, a smooth density gradient is formed in the centrifuge tube due to the polydispersity in size of the colloidal silica particles, which therefore sediment at different rates. In the present experiments free noradrenaline (NA) was found to bind to the fraction of the Ludox particles which accumulated at the bottom of the gradient. Extragranular amines were thereby removed from the solutions.

The experiments were generally carried out with mixtures of 6 ml of Ludox, 4 ml of sucrose (0.5 M, 1.0 M or 2.0 M) or distilled water and 1.5 ml of sample (see below). HCl had previously been added to the Ludox to adjust the pH to 8.0—8.2, and EDTA (0.5×10^{-4} M) to bind contaminating metal ions. The final Ludox concentration in the gradient solution was 25 % (w/w). The gradients were then generated in 12 ml polypropylene tubes fitting the Ti 50 angle head rotor by centrifugation in a Beckman L2 65 B centrifuge at 50 000 rpm for 30—60 min. In a few rat heart experiments the Ludox gradients were prepared in advance and the sample was then layered on top of the gradients and centrifuged. The gradients were usually fractionated with a Pasteur pipette, or in some cases by constant rate infusion of undiluted Ludox through a perforation in the bottom of the tube which lifted the gradient through a glass funnel into a polyethylene tubing which drained into collecting test tubes. The density of the different particulate fractions was measured in a petroleum/chloroform gradient as described by Oster (1965).

Preparation of tissue homogenates

Bovine adrenal glands and splenic nerves were obtained at the slaughter house about 20—30 min *post mortem* and were immediately chilled with ice.

The adrenal medulla was dissected out and homogenized in a Potter—Elvehjem apparatus with a teflon pestle in 5 volumes of isotonic sucrose (0.25 M). The homogenate was centrifuged at $800 \times g_{max}$ for 10 min in order to get rid of coarse particles. In some experiments catecholamine granules were sedimented by centrifugation at $15\,000 \times g_{max}$ for 15 min, and the sediment was resuspended in 0.25 M sucrose (resuspended high speed sediment). The amount of homogenate used for the gradients corresponded to 0.04—0.1 g of tissue.

The splenic nerves were dissected free and desheathed. Amounts of 1—3 g were transferred to incubation vessels with 5 ml of Krebs solution containing ascorbate 20 µg/ml. After addition of 50 µc of H^3-dl NA they were incubated at 37° C for 30 min. The nerves were then repeatedly washed in ice cold Krebs solution, blotted dry, transferred to beakers with 2—5 ml of 0.25 M sucrose, minced with scissors and homogenized by an Ultra Turrax apparatus. Coarse particles were removed by centrifugation at $9000 \times g_{max}$ for 10 min and the supernatant, "low speed supernatant", was used for gradient centrifugation.

Rat heart. Male and female Sprague Dawley rats weighing 150—250 g were given an injection in the femoral vein of 50 µc of H^3-dl NA.

The animals were decapitated after 30 min. The hearts were quickly dissected out, washed in 0.25 M sucrose, minced and homogenized in 2 ml of 0.25 M sucrose, with a conical glass homogenizer (Kontes). Coarse particles were removed by centrifugation at $800 \times g_{max}$ for 10 min. The supernatant was used for gradient centrifugation. In some cases mitochondria were removed by centrifugation at $15\,000 \times g_{max}$ for 15 min and the supernatant which contained most of the amine granules was analysed in the gradients.

Determination of radioactivity, catecholamines and cytochrome c oxidase

Aliquots of the different extracts were counted in a 7 : 3 toluene : absolute ethanol solution containing 4 g of 2,5 diphenyloxazole and 100 mg of 1,4 bis-2(5-phenyloxazolyl) benzene per litre of toluene in a Packard Tri Carb Liquid Scintillation Spectrometer. The counting time was 10 min. Quenching was monitored by internal standards.

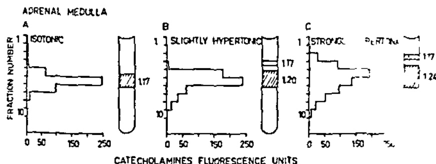


Fig 1 ... of adr ... and 4 ... B sl g ... The s ... were fractioned in 1 ml fractions for determinations of CA The figures to the right of the tubes refer to the density of the visible bands Abscissa CA (adrenaline and noradrenaline) in fluorescence units (F U) 200 F U corresponds to approximately 100 μg of CA

Catecholamines were determined fluorimetrically according to Euler and Lishajko (1961)

Cytochrome c oxidase activity was measured polarographically at 30° C using a Clark oxygen electrode, as described by Sottocasa *et al* (1967) The reaction mixture contained in 3 ml water 0 1 mM cytochrome c 16 mM ascorbate and 75 mM phosphate buffer pH 7 5 The reaction was initiated by addition of ascorbate

Results

Adrenal medulla

Four types of gradients were used hypotonic (addition 4 ml of distilled water) isotonic (addition 4 ml of 0 5 M sucrose), moderately hypertonic (addition 4 0 ml of 1 0 M sucrose) and strongly hypertonic (addition 4 ml of 2 0 M sucrose) In the hypotonic and isotonic gradients only one distinct particulate band was found, at a density level of about 1 17 g/cm^3, *i e* of the same density as 1 3 M sucrose This band contained both the CA granules and the mitochondria (Fig 1 A) In the hypertonic gradients (Fig 1 B and 1 C) two visible bands were formed, as in sucrose gradients The CA peaked in the denser band The less dense band probably contained the mitochondria as indicated by preliminary observations of cytochrome c oxidase activity

Splenic nerve

In isotonic gradients the amine particles from bovine splenic nerves formed a single band at the same level as that of isotonic medullary granules 1 17—1 18 g/cm^3 (Fig 2) The nerve granules did not change this position when the gradients were made hypertonic as did the medullary granules

Rat heart

The peak of the NA radioactivity was found at the same level as that of the splenic nerve granules, at 1 17—1 18 g/cm^3 The cytochrome c oxidase activity formed a

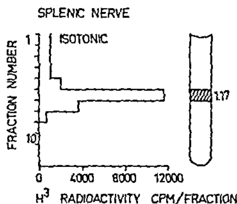


Fig 2 *Subcellular distribution H³ noradrenaline in bovine splenic nerves* The splenic nerves were incubated with H³ noradrenaline as described in text Supernatant of a homogenate centrifugated at 9000×g for 10 min ... adrenaline)

peak at about the same level (Fig 3) However, an additional small peak of labeled NA was found in every gradient (7 expts) at a density level of 1 07 g/cm³, i e the same density as 0 5 M sucrose In one rat heart experiment the homogenate was layered on a preformed silica gradient and centrifuged for 2 hrs at 50 000 rpm Even here a small peak at the corresponding level of 0 5 M sucrose could be seen The possibility that the apparent density of the 'heavy' nerve granules could be due to aggregation of amine granules with mitochondria was also studied In this case the mitochondria were removed by centrifugation at 15 000×g_{max} for 15 min and the supernatant was run in a Ludox gradient The radioactivity was found to have about the same distribution as in the previous experiments with a double NA peak (cf Fig 3)

Discussion

As mentioned in the Introduction the available information in the literature concerning the density of CA storage particles from adrenal medulla and sympathetic nerves is rather confusing Methodologically the reports are based on analysis by centrifugation on sucrose density gradients in which the graded densities are produced by raising the concentration of sucrose Obviously, this technique produces

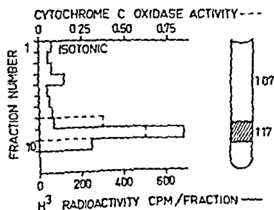


Fig 3 *Subcellular distribution of H³ noradrenaline and cytochrome c oxidase in the rat heart* Supernatant of a rat heart homogenate (incubated and prepared as described in the text) was mixed with Ludox and with sucrose to produce a final tonicity of about 3... mosmol/l *Centrifugation* 50 000 rpm for 40 min Upper abscissa Cytochrome c oxidase activity (moles of O_2/min) *Lower abscissa* Radioactivity (mainly H³ noradrenaline)

at the same time an osmolarity gradient The puzzling differences in apparent density of these particles might thus be artifactual and due to differences in the degree of osmotically induced shrinking (*cf* de Duve 1965), which all other factors being equal should be a function of the volume/surface ratio of the particles (*cf* Potter 1967) Alternatively an increased density could be the result of selective sucrose penetration into the particles In order to clarify the issue it seemed necessary to repeat the analysis by centrifugation in gradients in which the graded densities can be obtained without changes in osmolarity and where the supporting medium is of macromolecular nature

The silica solution used in the present experiments provides a suitable tool for this kind of analysis (*cf* under Methods) In the silica gradient the osmolarity is largely uniform throughout and can be adjusted at will by addition of sucrose to hypotonic isotonic or hypertonic levels

When centrifuged essentially to equilibrium in this type of gradient the banding characteristics of the amine granules from bovine adrenal medulla were found to vary with the tonicity of the medium Thus in hypotonic as well as in isotonic gradients the granules formed a single band at a relatively low density level close to that of the mitochondria However in silica gradients deliberately made hypertonic by addition of sucrose the granules formed at band distinctly separate from the mitochondrial one at the same density level at which the granules are found in ordinary sucrose gradients Apparently the average density of the amine granules increases markedly if they become dehydrated and shrink in the hypertonic medium Thus the separation of amine granules from mitochondria obtained by sucrose density gradient centrifugation is probably not due to true differences in density between these particles but rather to the fact that the amine granules change their density more in hypertonic medium than do mitochondria Similar results have recently been reported by Laduron (1969) who used density gradients made of glycogen and sucrose

Regardless of the osmolarity of the medium splenic nerve granules analyzed by the same system were found to form a single band with a maximum at about the same density level as that of the mitochondria and of the adreno-medullary granules in isotonic gradients This apparent density value for splenic nerve granules is in agreement with previously observations in sucrose gradients (Roth *et al* 1968)

Most of the rat heart granules also sedimented to this level and did thus not by this method separate very well from the mitochondria However in all experiments a small fraction of the NA radioactivity was found to form a sedimentation maximum at a considerably lower density level The constant duplicity of the amine peak in the rat heart is of interest in view of previous observation of two different particulate NA peaks in sucrose density gradient analysis of this tissue (Roth *et al* 1969) How e

The possibility ...
existence of two different population of NA particles However it is not possible at

present to exclude that the apparent duplicity is artifactual

Thus in conclusion, it appears that the repeated demonstration of striking differences in apparent density between CA granules obtained by sucrose density gradient centrifugation from sympathetic nerves and chromaffin tissue may be to a large extent artifactual The present observations indicate that the "true" density of chromaffin granules may be close to that of the majority of nerve granules On the other hand, the results also give some support to previous evidence for the existence of at least two different populations of nerve granules in tissues like the rat heart with different banding properties Finally, the present results of density gradient centrifugation analysis in an isotonic system emphasize that the osmotically induced artifactual changes in the density of chromaffin granules in combined density and osmolarity gradients may be purposely utilized for separation of CA granules from other intracellular organelles, such as mitochondria, from which the granules do not separate as well in isotonic gradients In this connection it is interesting that D_2O gradients can be used for the same purpose since D_2O penetrates into the granules more easily than into mitochondria (*cf* Laduron 1969) This possibility was recently utilized by Poisner and Trifaro (1969) who obtained good separation of chromaffin granules from mitochondria by using isotonic Ficoll-sucrose-D_2O density gradients

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Cyclic Variation in the Secretion of Seminal Fructose in Rabbits

By

G DEGERMAN AND J E KIHLSTROM

Received 16 September 1969

Abstract

DEGERMAN G and J E KIHLSTROM *Cyclic variation in the secretion of seminal fructose in rabbits* Acta physiol scand 1970 *78* 567–570

A cyclic variation of the level of seminal fructose in rabbits has been demonstrated by the use of power spectrum analysis As the production of this substance is regulated by the testicular hormones also the activity of these hormones is supposed to vary cyclically

According to the current concept only the female sexual functions are submitted to cyclical variations while the male ones are not (Bullough 1951) However cycles in males of approximately the same length as that of the oestrus cycle of the corresponding females have been demonstrated in sexual functions in rabbits (Doggett 1956 Kihlstrom 1958) cattle (Kihlstrom 1962) rats (Kihlstrom 1965) sheep (Tischner 1967) and mice (Jarnebrand and Kihlstrom 1969), and occur possibly also in man (Doggett and Keilers 1962 Exley and Corker 1965, 1966 Halberg *et al* 1965 Hornstein 1965 Månsson 1965) A review of literature published in this field up to 1965 was given by Kihlstrom (1966)

It might be suspected that all these cyclic phenomena are different expressions of a corresponding fluctuation in the hormonal status most likely in the circulating quantities of male sexual hormones (Degerman and Kihlstrom 1962 Hornstein Kihlstrom and Degerman 1964) Unfortunately, the methods so far available for the quantitative determination of testosteron require amounts of blood too large to be withdrawn frequently enough for studying cycles with a duration of a few days However the level of fructose in the seminal plasma is known to be intimately dependent upon the testicular hormones (Mann and Parsons 1947) The variation from day to day in the secretion of seminal fructose in rabbits might therefore give some information concerning the hormonal background of these cycles

Material and Methods

8 sexually mature male rabbits of different breeds none of them more than 2 years old were studied from the middle of January to the beginning of May The animals were kept in separate cages in a windowless room and subjected to an artificial diurnal rhythm with a light period extending from 8 a m to 8 p m They were all given the same kind of food once a day between 9 and 10 a m The temperature of the room was thermostatically regulated and varied between +16 and +18° C Semen was collected by means of an artificial vagina once every day between 8 and 9 a m, before the start of the ordinary routine in the stable The ejaculates were collected in test tubes With this method a small part of each ejaculate

[illegible]

for small amounts as follows 50 µl of the seminal plasma were transferred to a (volume 1 0 ml) containing 200 µl distilled water 300 µl of a 6 7 % solution of $ZnSO_4 \cdot 7H_2O$ were added followed by 200 µl 0 5 M NaOH, this sequence of the additions being of great importance The contents of the tube was well mixed before the next component was added After heating the tubes on a boiling water bath fo tap water the protein precipitate was spun down natant were mixed in a test tube (volume 20 ml) acid The solution thus obtained could then be sto changing After thawing 2 0 ml of a solution 5 0 ml HCl (30 % solution) were added The contents of the tube were well mixed the tube heated in a thermostatically regulated water bath at +85° C for exactly 8 min and immediately chilled in ice water The colour was fully developed after 15 min and remained stable for at least 7 hrs provided that the tubes were kept at 0° C The extinction was measured at 485 nm By the use of known solutions of fructose instead of seminal plasma a standard curve was obtained This curve is linear within a range corresponding to 1 0 to 10 5 µg fructose/ml of the sample With the dilutions performed this corresponds to 0 3—3 4 mg/ml seminal plasma

The power spectrum analysis according to Blackman and Tukey (1958) was used for the statistical treatment of the data obtained This method is looked upon as the most suitable for the study of biological time series A power spectrum is a diagram giving the variance as a function of the freque will appear as a signi In analogy to similar the name power spectrum analysis An electronic computer was used for

Results

The results are presented in Table I as the width in days of the bands of the power spectrum demonstrating statistically significant preference

Concentration of fructose The mean of all 779 determinations is 1 63 mg/ml with a standard deviation of ±0 55 As seen from the table a statistically significant cyclic variation could be demonstrated in all the studied animals Assuming the midpoint of each band demonstrating significant preference to represent the real length of the cycle we find a mean length of 6 5 days

Absolute content of fructose ... cycles are The mean value is 0 84 ± 0 40 (S D) ... s the most demonstrated by 5 out of 8 animals representative value we find a mean length of the cycles of 6 3 days

In a few cases a short cycle seems to be superimposed by a longer one the length of which might be a multiple of that of the former

TABLE I Cyclic variation in some seminal characteristics in rabbits ejaculating once every day

Rabbit No	*Concentration of fructose* Width in days of the bands of the spectrum demonstrating statistically significant preference	p	*Amounts of fructose per ejaculate* Width in days of the bands of the spectrum demonstrating statistically significant preference	p	*Volume of the ejaculates* Width in days of the bands of the spectrum demonstrating statistically significant preference	p
1	8 0— 6 2	<0 05	8 0—6 2	<0 05	3 3— 2 9	<0 01
5	6.2— 5 1	<0 05	6 2—5 1	<0 05	8 0— 6 2	<0 01
6	8 0— 5 1	<0 05	—		8 0— 6 2	<0 01
7	18 7— 8 0	<0 05	8 0—6 2	<0 05	—	
			2 9—2 7	<0 05		
8	5 1— 4 3	<0 05	—		—	
9	18 7—11 2,	<0 05	—		8 0— 6 2	<0 01
	3 7— 3 3	<0 05				
10	18 7—11 2,	<0 05,	11 2—6 2	<0 05	18 7—11 2	<0 005,
	3 7— 3 3	<0 05			11 2— 8 0	<0 005
11	8 0— 6 2	<0 05	8 0—6 2	<0 005	8 0— 6 2	<0 01

Besides the power spectrum analysis reveals that the volumes of the ejaculates vary cyclically (Table I), which confirms earlier observations (Kihlstrom 1958 Degerman and Kihlstrom 1961)

Discussion

The observed levels of seminal fructose correspond well with the data given in the literature (Mann 1964)

Apparently there are strong reasons to believe that the production of fructose in the accessory sex glands of male rabbits varies cyclically As the production of this substance is regulated by the testicular hormones, the present results may indicate a cyclic variation in the circulating amounts of these hormones The same conclusion has been arrived at also in other investigations Thus in rabbit semen the occurrence of the gelatinous substance the production of which is known to be regulated by androgens (Parsons 1950) varies cyclically (Kihlstrom and Degerman 1962 Moreover the cyclic variation in the exfoliation of cells from the male urethra of the rabbit disappears after castration of the animals (Hornstein Kihlstrom and Deger

ing a cyclic

and Corker

s of type C

having an androgen dependent nuclear appendage varies cyclically (Månsson 1965)

The statistical analysis reveals a more significant cyclicity in the volumes

ejaculates than in the level of fructose in the seminal fluid This fact may indicate that the secretion of a certain amount of fructose is independent of the testicular hormones and superimposed by a varying, hormone dependent secretion Moreover rabbit semen occasionally has an admixture of glucose (Mann and Parsons 1950) and the method used is not quite specific for fructose An irregular addition of glucose may thus be another explanation of the comparatively low significance

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Experimental Hypothermia in Neonatal Lambs Poor Cardiac Response to Increased Oxygen Consumption during Shivering

By

ANTTI KOIVIKKO

Increased oxygen consumption due to different causes is mostly followed by an increased cardiac output in adult mammals Also arterial hypoxia increases the cardiac output of mammals, but the cardiac output response of neonatal lambs to arterial hypoxia is much weaker than that of adult sheep (Cross *et al* 1959 Koivikko 1969) As shivering during hypothermia is a condition during which oxygen consumption increases, this experimental condition was chosen to investigate the cardiac response of neonatal lambs to increased oxygen consumption

The animal material comprised 13 lambs 1 to 7 days old 9 of them were anesthetized with chloralose (30 to 50 mg/kg) to prevent shivering and four were given the anesthetic for sedation only (20 to 50 mg/kg) and were allowed to shiver

Cardiac output stroke volume heart rate and central blood volume were measured by the dye dilution method and by recording ECGs The body temperature was recorded with an esophageal electrode Hypothermia was produced by cooling the blood by pumping it continuously through the ice water bath with a roller pump Oxygen consumption was measured with an animal spirometer

Results and Discussion

It will be noted that the oxygen consumptions (Fig 1) of the shivering animals (A B and C) remained high during hypothermia or increased above the initial level whereas an exponential decrease of oxygen consumption occurred in the animals that did not shiver Lamb D presents a borderline case it shivered visibly but its oxygen consumption did not increase possibly owing to the fact that it had been given 50 mg/kg of chloralose These results are in general similar to those obtained with adult mammals (Gribbe *et al* 1961 and 1964)

Fig 2 shows that the cardiac outputs of the non shivering lambs decreased exponentially during hypothermia This decrease was 30 to 50 per cent more (when the temperature decreased from 36 to 27° C) than that observed in Nembutal-anesthetized dogs and cats by Gribbe *et al* (1961, 1964) The cardiac outputs of

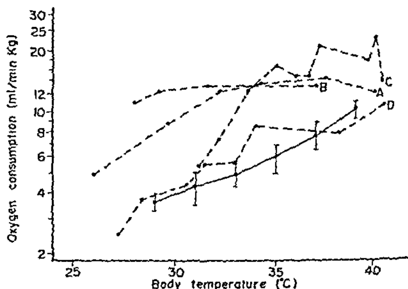


Fig 1 Oxygen consumption in relation to body temperature The solid line refers to the lambs which did not shiver and broken lines to the shivering lambs

lambs A, B, and C decreased to the same extent as those of the non-shivering animals although their oxygen consumptions increased This observation differs from the finding of Prec *et al* (1949) that both oxygen consumption and cardiac output increased when blood was cooled to 30° C in shivering adult dogs In the present series there occurred only one slight increase of the cardiac output at the beginning of the cooling period (lamb C)

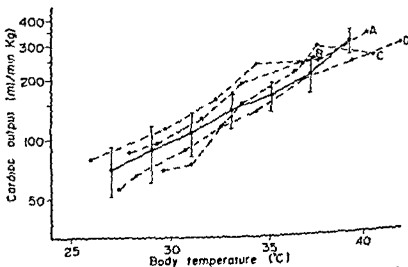


Fig 2 Cardiac output in relation to body temperature For explanation see the text of Fig 1

This unresponsiveness of cardiac output to increased oxygen consumption in the lambs may be related to the poor cardiac output response of lambs to arterial hypoxia as compared with that of adult sheep (Cross *et al* 1959 Koivikko 1969) Although the arterial oxygen saturation was kept over 97 per cent by oxygen respiration in the present study, there is no doubt that the oxygen tension of the tissues decreased owing to increased A—V difference Evidence has been presented which shows that the cardiac output response to arterial hypoxia depends on the degree of metabolic acidosis (Koivikko 1969) Increasing degrees of metabolic acidosis were observed in some shivering lambs as the body temperature decreased The role of this and other factors which may influence the cardiac response remain to be investigated in detail

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Failure of Growth Hormone to Exert an Acute Inhibitory Effect on Glucose Uptake in the Rat Diaphragm

By

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It is well known that the pituitary growth hormone (GH) has marked effects not only on the protein metabolism of the body but also on carbohydrate and lipid metabolism. The physiological significance of the GH effects on carbohydrate and lipid metabolism is however still unclear.

Most investigators agree that the various effects of GH develop in a specific order both after *in vivo* and *in vitro* administration of the hormone. The early effects which occur within the first 1—2 hrs after the administration of the hormone are those effects which favour increased utilization of glucose and the storage of fat and these effects have been referred to as "stimulatory" or "insulin like". The late or "inhibitory" effects of GH are not generally seen until 2—3 hrs after the administration of the hormone. One characteristic feature during this late phase is a decreased utilization of glucose.

It was therefore rather surprising when Bolodia and Young (1967) reported that addition of very small concentrations of GH to the isolated diaphragm muscle from hypophysectomized rats produced an acutely decreased uptake of glucose. When they added higher concentration of the same GH preparation to the isolated diaphragm they found the same increase of glucose uptake as other investigators have repeatedly observed. Bolodia and Young concluded that the acute inhibitory effect on glucose uptake of very small amounts of GH might represent the physiological role of the hormone.

We have in this laboratory long been interested in early and late effects of GH on the rat diaphragm muscle (e.g. Hjalmarson 1968) and we have always found increased uptake of glucose, non utilizable sugars and amino acids as early effects of the hormone. We have, however, never tried such low concentrations of GH as those used by Bolodia and Young (1967). We have therefore repeated some of our earlier experiments, now using the same low concentrations of GH as those reported by Bolodia and Young (1967).

Female rats of the Sprague Dawley strain were used. Hypophysectomy was performed at the age of 4—5 weeks and the rats were used 12—16 days later for the *in vitro* experiments. The "cut" hemidiaphragms first described by Gemmill (1940) and then used by many investigators (e.g. Bolodia and Young 19[illegible]) were used when glucose uptake and the incorporation of glycine-[illegible] ^{3}H (0.1 mM, 225 µC[illegible]) into the protein was studied. The "intact" hemidiaphragm [illegible]

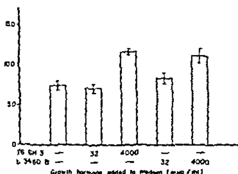


Fig 1

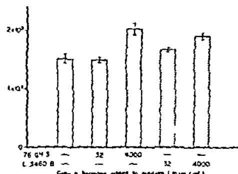


Fig 2

Fig 1 Effects of two preparations of bovine growth hormone (76 GH3, L 3460 B) *in vitro* on the uptake of glucose by cut diaphragms from hypophysectomized rats Glucose uptake is expressed as mg of glucose taken up per g of muscle (wet weight per 2 hrs The standard error of the mean is indicated at the top of each column Both preparations of growth hormone produced a significant stimulatory effect when added to the medium at a concentration of 4000 mμg/ml ($p < 0.02$) No significant effects were seen when the hormones were present at a concentration of 32 mμg/ml

76 GH3 L 3460 B *in vitro* on ophysectomized M per 100 μg d at the top of imulatory effect ificant effects

phragms was determined as described in earlier papers (*e g* Hjalmarson 1968) The rate of ... was calculated ... ween the radio- tion period, ex determined and

bach L 3460B batch 76 GH 3

The results are summarized in Fig 1 and 2 It can be seen from these figures that none of the growth hormone preparations used influenced glucose uptake or incorporation of glycine-³H into the protein of the cut hemidiaphragm preparation when the hormones were present in a concentration of 32 mμg/ml medium *i e* the concentration that reduced the glucose uptake in the experiments of Bolodia and Young (1967) It can also be seen from these figures that addition of the two growth hormone preparations in concentrations of 4 μg/ml medium stimulated both glucose uptake and glycine-³H incorporation

In the experiments with the intact hemidiaphragm preparation the AIB upt was not significantly changed by the addition of 32 mμg/ml medium of the growth hormone preparations Addition of the hormone in a concentration of 4 ml medium markedly stimulated the AIB uptake, the distribution ratios were t for the controls 0.88 ± 0.08, and for the two GH preparations 1.81 ± 0.12 a 1.83 ± 0.15

The above mentioned results were from experiments on diaphragms from hy physectomized rats which had not been deprived of food for any time before acute experiments The same results were in principal however, received in expe ments on diaphragms from hypophysectomized rats deprived of food for 24 hrs fore the experiments

It was thus under our experimental conditions not possible to detect any acute hibitory effect of growth hormone on glucose uptake in the diaphragms of hyp physectomized rats Only stimulatory effects on glucose uptake on incorporation glycine into protein and on intracellular accumulation of AIB were seen in acco ance with many earlier reports from this and other laboratories We have at prese no clear explanation of the discrepancy in results between our experiments and the of Bolodia and Young (1967) Small differences in the experimental procedures in the growth hormone preparations used might be the explanation Further studi with various growth hormone preparations performed in different laboratories a therefore necessary to elucidate this point The question whether the pituitary grow hormone has any acute inhibitory effects on glucose lipid or protein metabolism i however of such importance that also negative findings such as those of the prese study are important to report

We wish to thank Professor C H Li and Professor F G Young for their generous gift of t growth hormone preparations Valuable technical assistance was given by Miss Elisabet Johan son

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